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**GENOMIC INSTABILITY IN SPORADIC  
COLORECTAL CANCER**

**Lucy Jane Curtis**

**Thesis submitted for the Degree of  
Doctor of Philosophy**

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## **DECLARATION**

I declare that this thesis was composed entirely by myself and that the work presented is my own unless otherwise stated.

A handwritten signature in black ink, appearing to read 'Lucy J. Curtis', with a stylized, cursive script.

Lucy J. Curtis

March, 1998.



## ABSTRACT

Genomic instability is fundamental to the development of human neoplasia. This thesis has examined aspects of two forms of instability prominent in sporadic colorectal cancer, microsatellite instability (MSI) and chromosomal instability. Microsatellite instability describes a phenotype in which mutation of microsatellite sequences is widespread, probably due to defects of components of the mismatch repair pathway. Clonal chromosomal abnormalities, frequently observed in sporadic colorectal cancer, probably arise because of instability at the chromosomal level, and there is substantial, though not conclusive, evidence that they may result from defects of the p53 protein.

Several lines of evidence suggest that chromosomal instability and defects of p53 may represent a mechanism, or mechanisms, of tumorigenesis distinct from that driven by defects of the mismatch repair system. In this project, a series of experiments were undertaken to investigate the microsatellite instability phenotype, its possible cause, and the extent to which it coexists and interacts with chromosomal instability and p53 abnormalities. MSI was examined in a large population of sporadic human colorectal cancers and related to clinical and pathological tumour features in order to define traits which may suggest a distinct biological basis. Such instability was found to confer significant survival advantage, and to be inversely related to abnormality of the p53 protein, supporting the notion that these defects represent distinct tumorigenic pathways. In an attempt to discover the cause of microsatellite instability, mutation analysis of a likely candidate gene, hMSH2, was undertaken in selected cases. This analysis yielded few exonic gene mutations, supporting data from other studies which suggest that hMSH2 is not an important cause of the MSI phenotype in sporadic colorectal cancer. To further investigate the MSI phenotype, the behaviour of stable and unstable microsatellites over a period of time was examined through use of a series of xenografted human cancers. This demonstrated maintenance of the primary tumour phenotype in all xenografts over several months' passage *in vivo*, adding weight to the argument that microsatellite instability is persistent and is driven by an underlying retained defect.

To define unique patterns of chromosomal anomalies associated with MSI and defects of p53, xenografted human colorectal cancers were analysed by Comparative Genomic Hybridisation (CGH). Cancers without MSI displayed a striking pattern of chromosome change in almost all cases, involving combinations of deletion of 8p and 18q and duplication of 8q, 13q and 20q, suggesting that clonal selection for genes of importance in these regions is important in such cancers. In contrast, cancers with MSI did not display this pattern, and

were associated with a decreased number of chromosomal gains. These data support the hypothesis that (at least) two major mechanisms of oncogene and tumour suppressor gene disruption exist. The relationship between p53 abnormalities and chromosome changes was less clear, though a trend towards high levels of chromosomal anomalies in tumours with defective p53 was seen. This study was extended by similar analysis of tumours taken from mice lacking *p53* and/or the mismatch repair gene *Msh2*. Tumours lacking all p53 function usually had high levels of chromosomal defects, whereas those lacking *Msh2* had few. When both functions were removed, the phenotype of few chromosomal changes and extensive microsatellite instability predominated, suggesting defective mismatch repair to be the dominant pathway of instability in clonal selection during tumorigenesis. The data presented here provide insight into the nature, co-existence and interaction of different forms of genomic instability in cancer, which may ultimately help our understanding of the heterogenous nature of colorectal cancer and facilitate appropriate design of new and existing chemotherapeutic regimens.



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# CHAPTER 1

## GENOMIC INSTABILITY IN COLORECTAL CANCER

### 1.1. Introduction

This thesis is about the mechanisms of genomic instability which underlie the initiation and progression of sporadic colorectal cancer. These processes are fundamental to neoplastic development, and many genetic defects which have long been recognised as crucial events in colorectal tumorigenesis occur as a direct result of genomic instability. Still others generate and sustain such instability. Thus, the genetic defects in colorectal cancer can be better understood in the context of the dynamic processes underlying the degeneration of the cancer genome. To introduce this, this chapter gives an overview of current knowledge of the genetic events and environmental influences which play a role in sporadic colorectal tumour development. The specific types of genomic instability pertinent to this study and the mechanisms which may cause them, namely microsatellite instability, defects of the mismatch repair pathway, anomalies of the p53 protein and chromosomal lesions, are discussed. Studies of sporadic cancer have been aided by increasing knowledge of hereditary disease, and genetic events in inherited cancer are considered in relation to sporadic cancer. Finally, the aims and objectives of this project are presented, together with a brief summary of the chosen methods.

## 1.2. Clinical aspects and aetiology of colorectal cancer

### 1.2.1. Incidence and distribution of colorectal cancer

Cancer of the large bowel is the second most common cause of cancer death in Westernised countries, accounting for 19000 deaths each year in the UK. Incidence varies considerably worldwide, one of the highest rates being in Scotland, at 20.5/100 000 males in 1991 (Cancer Research Campaign Factsheet 18.1 & 18.2, 1993). Incidence is increasing in low-risk populations, such as Mediterranean countries, Central and South America, Asia and Eastern Europe, as lifestyles become increasingly Westernised (Weisburger, 1991). Risk of developing colorectal cancer increases with age; the likelihood of developing cancer at age 30-34 is small (2.9/100 000) compared with the risk at age 85 or older (531.6/100 000) (Levin & Dozois, 1991).

### 1.2.2. The adenoma-carcinoma sequence

Adenocarcinoma accounts for more than 90% of colorectal cancers. Several lines of evidence support the hypothesis that many carcinomas derive from a premalignant dysplastic lesion, the adenoma. Firstly, population distribution of adenomas reflects that of carcinomas, and populations acquiring increased risk of colorectal cancer through moving to areas with high cancer incidence also acquire an increased risk of developing adenomas (Weisburger, 1991). Approximately 70% of all cancers occur distally within the bowel, within the sigmoid colon, descending colon and rectum. The distribution of adenomas is similar, with an age of onset approximately 5 years earlier (Muto, 1975), consistent with sequential progression. Finally, histological examination shows that adenoma and carcinoma tissue are often contiguous: foci of carcinoma can be found within adenomas, and islands of residual adenoma can be found within carcinomas (Lane, 1976). Although the progression from adenoma to carcinoma is thought to occur in the vast majority of Western colorectal cancers, it is possible that 'de novo' cancers sometimes arise without preexisting adenoma. In particular, it has been suggested that cancers with flat morphology very often arise de novo in the Japanese population (Wada *et al.*, 1996), though this is disputed (Owen *et al.*, 1996).

Hyperplastic polyps, unlike dysplastic adenomas, are believed to be uninvolved in the development of colorectal cancer (Lane, 1976), and recent molecular evidence supports this notion. Mutation of the adenomatous polyposis (APC) gene (discussed in 1.3.1) is very common in all dysplastic lesions, microscopic aberrant crypt foci (ACFs), adenomas and carcinomas, but rare in hyperplastic polyps (Jen *et al.*, 1994a). In contrast, all hyperplastic ACFs can be shown to harbour mutations of the Ki-ras oncogene (discussed in 1.3.4), whilst the incidence of Ki-ras mutations in all other tumours is much lower. This suggests that hyperplastic ACFs are not the precursors of either dysplastic or non-dysplastic colonic tumours, whereas dysplastic ACFs could well precede adenoma and carcinoma formation.

### 1.2.3. Treatment and prognosis of colorectal cancer

Prognosis for this disease is relatively poor, with about one third of all patients surviving to 5 years after diagnosis (Cancer Research Campaign factsheet 18.1, 1993). The primary treatment remains surgical, often in conjunction with radiotherapy for control of local recurrence of rectal cancer. Adjuvant chemotherapy is used for control of occult liver metastases, but colorectal carcinoma is relatively refractory to chemotherapy and administration of such treatment affords only a small increase in survival of about 10-15% (International multicentre pooled analysis of colorectal cancer trials investigators, 1995). Most regimens employ the anti-metabolite 5-fluorouracil (5-FU) in combination with a secondary factor such as folinic acid or levamisole. Prophylactic administration of aspirin and other non-steroidal anti-inflammatory drugs (NSAIDS), which have been demonstrated to have a strong protective effect against colorectal cancer in mice and man (Labayle *et al.*, 1991; Boolbol *et al.*, 1996), is currently under investigation.

Histopathological staging is still described according to Dukes' classification of 1932, and Dukes' stage remains the main histopathological predictor of survival (Newland *et al.*, 1981; Wiggers *et al.*, 1988; Laurent-Puig *et al.*, 1992). In addition, several genetic factors, discussed in the text below, have a significant influence on prognosis. Notably, relatively poor 5-year survival is associated with alterations of the p53 gene and certain chromosomal alterations and deletions, whilst several studies indicate improved survival in patients whose cancers have a diploid karyotype and in patients whose tumours demonstrate microsatellite instability.

### 1.2.4. Dietary factors and sporadic colorectal cancer

Diet is a major aetiological factor in colorectal cancer, and is important to consider in this discussion because the nature of genetic and cellular damage initiated by dietary components may determine subsequent genetic events. Several dietary factors have been demonstrated to be associated with increased risk of colorectal tumours. Principally, an increased risk of cancer is associated with a diet high in saturated fat and low in fibre, though the type of fat and fibre consumed is important (see Weisburger, (1991) and Reddy, (1993) for reviews; Little *et al.*, 1993; Olsen *et al.*, 1994). Intake of calcium and certain vitamins may also reduce risk (Weisburger, 1991; Olsen *et al.*, 1994). Alcohol, especially beer intake, is associated with increased risk of rectal carcinoma (Weisburger, 1991; Cancer Research Campaign Factsheet 18.1, 1993). Studies in mice suggest that, although both dietary fat and caloric intake affect tumour growth, the effect of caloric intake may be more profound (Sarkar *et al.*, 1995).

Little is understood about the mechanisms by which dietary components affect cellular proliferation. Some dietary constituents and products of digestion are directly

genotoxic, whilst others may influence cellular processes such as apoptosis which affect the rate of tumour initiation or progression. One example of a dietary component thought to be directly damaging to DNA is the heterocyclic amine 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhiP), present in some cooked food. PhiP induces colon tumours in rats in which, in common with human colonic cancers, the APC gene is often truncated (Kakiuchi *et al.*, 1995). However, the characteristic mutagenic 'fingerprint' of PhiP (Yadollahi-Farsani *et al.*, 1996) does not suggest its direct involvement in mutation of APC in a large number of human cancers. In contrast, bile acid, which increases as a result of high fat intake, is cytotoxic, producing a high cell turnover and associated risk. Furthermore, bacterial degradation of bile salts may result in production of carcinogens in the bowel lumen, so carcinogen production from this process could be increased either by alteration in the bacterial population by changes in dietary fibre and carbohydrate or by increased bile acid production (Burkitt, 1971). One study indicates that caloric restriction slows tumour growth in mice through increasing the apoptotic rate through reduction of insulin-like growth factor I (Dunn *et al.*, 1997).

The overall carcinogenic effects of diet are partially governed by individual genetic profile, either in terms of abnormalities in genes predisposing to cancer, discussed below, or more subtle polymorphisms in metabolic enzymes. For example, heterocyclic amines, which can be present in the diet, are activated by a pathway involving N-acetyl transferase, and individuals with rapid acetylation phenotype are at increased risk of colorectal cancer (Ilett *et al.*, 1987). A polymorphism of CYP1A1, an enzyme of key importance in the metabolism of polycyclic aromatic hydrocarbons, is associated with increased colorectal cancer risk (Sivaraman *et al.*, 1994). Similarly, individuals with bowel cancer have a slightly higher than usual incidence of the null genotype of the GSTM1 locus, the product of which normally detoxifies polycyclic aromatic hydrocarbon metabolites (Zhong *et al.*, 1993). Patients with proximal bowel cancer have a significantly increased incidence of the GSTM1 null allele, suggesting a difference in the importance of this enzyme between the left and the right colon.

Finally, the mode of action of the *mom1* locus (modifier of *min*) illustrates the complexity of interaction between diet and genetic complement which is just beginning to be elucidated. This is discussed in 1.3.1.1.

#### 1.2.5. Predisposition to colorectal cancer

Several diseases confer an increased risk of bowel cancer. Up to 25% of ulcerative colitis patients eventually develop the disease (Cotran *et al.*, 1994) and, similarly, Crohn's disease confers a cancer risk 4 to 20 times that of the general population (Levin & Dozois, 1991). However, by far the most dramatic risk is conferred by the inherited cancer predisposition syndromes Familial Adenomatous Polyposis (FAP) and Hereditary Non-

Polyposis Colorectal Cancer (HNPCC). These syndromes are described in the following discussion of colorectal cancer genetics.



### 1.3. The genetics of colorectal cancer

The proliferation of a population of cells is regulated by both tumour suppressor genes, which inhibit cell growth or promote death, and by proto-oncogenes, which promote growth or inhibit death. Loss-of-function mutations of tumour suppressor genes or activating mutations of proto-oncogenes (to create oncogenes) creates deregulation of growth control, leading to the rate of cell proliferation surpassing the rate of cell death and subsequent tumour formation.

Whilst oncogenes are dominantly-acting elements, tumour suppressor genes are recessive at the cellular level and often require inactivation of both alleles in order to exert an effect on cellular growth. A model for tumour suppressor gene function was developed on the basis of observations of retinoblastoma, a rare childhood tumour of the eye (Knudson, 1971). According to this model, known as Knudson's 'two-hit' hypothesis, sporadic disease would only arise if two copies of the same gene were inactivated through mutation. However, children who inherited one mutated copy of a gene require only one somatic event in order for a tumour to develop.

Tumorigenesis in colorectal cancer is a multistep process characterised primarily by the loss of function of tumour suppressor genes and, less frequently, by the mutation and amplification of proto-oncogenes. Several genetic aberrations, summarised in Table 1 at the end of this chapter and Figure 1, and described in the following sections, appear frequently in colorectal cancer and it is widely accepted that an accumulation of lesions in critical genes is necessary for the progression to carcinoma (Fearon & Vogelstein, 1990; Kinzler & Vogelstein, 1996) [Figure 1]. Recent thought emphasises the dynamic processes involved in the acquisition of such genetic lesions, which may occur in a cascade following a single critical event such as a failure to respond to DNA damage or to initiate DNA repair. The critical target genes which acquire mutations in sporadic and hereditary colorectal cancer are thought to be largely, though not entirely (Markowitz *et al.*, 1995; Konishi *et al.*, 1996), similar, though the tumorigenic mechanisms through which the mutations occur can differ.

The remainder of this chapter describes the roles of the molecular events known to be critical in the process of colorectal carcinogenesis, with reference to their relationship to mechanisms of tumorigenesis.

### 1.3.1. The APC gene in Familial Adenomatous Polyposis and sporadic colorectal cancer

Elucidation of the genetic basis of Familial Adenomatous Polyposis (FAP) has been fundamental to understanding the early events occurring in sporadic colorectal cancer. FAP is an autosomal dominant familial cancer predisposition syndrome affecting around 1 in 7000 people. The disease is characterised by the development of hundreds of thousands of colorectal adenomatous polyps at an early age, some of which inevitably progress to cancer without surgical intervention. Many of these individuals are also prone to develop extracolonic manifestations such as congenital hypertrophy of the retinal pigmentosum endothelium (CHRPE), osteomata and desmoid tumours.

Individuals with FAP carry one mutated copy of the APC tumour suppressor gene (Groden *et al.*, 1991; Joslyn *et al.*, 1991; Kinzler *et al.*, 1991a; Nishisho *et al.*, 1991). Tumours develop when normal function of the remaining allele is lost (often through large deletion of a portion of the chromosome containing APC, or through point mutation) though only a few of the many benign lesions which develop progress to carcinoma. Thus, APC functions as a classical tumour suppressor gene according to Knudson's hypothesis (Knudson, 1971). Germ-line mutations of the gene in FAP patients are mainly confined to one half of the large terminal exon 15 (Miyoshi *et al.*, 1992a), and the vast majority cause truncation of the protein product. Loss of function of APC is thought to act as a 'gatekeeper' to tumorigenesis, after which an accumulation of other gene mutations characterise a relatively slow progression to metastatic disease (Kinzler & Vogelstein, 1996).

Evidence for the involvement of APC in sporadic colorectal cancer came initially from loss of heterozygosity (LOH) studies which demonstrated that the region of chromosome 5q containing APC was frequently deleted in these tumours (Solomon *et al.*, 1987; Vogelstein *et al.*, 1988; Ashton-Rickardt *et al.*, 1989 & 1991). Mutation analysis demonstrated somatic mutations in over 60% of tumours, even in very small sporadic adenomas (Miyoshi *et al.*, 1992b; Powell *et al.*, 1992) and dysplastic aberrant crypt foci (Jen *et al.*, 1994a), predominantly within a small 'mutation cluster region' of the largest exon of APC (Miyoshi *et al.*, 1992b). Mutation of APC is now believed to occur in almost all colorectal neoplasms (Miyoshi *et al.*, 1992a; Nagase & Nakamura, 1993), suggesting that the growth-regulatory feature of APC is important in preventing proliferation at the earliest stages of tumorigenesis.

The biochemical properties and biological functions of APC are not fully understood. The gene encodes a 300kD cytoplasmic protein expressed in the upper portion of the colonic crypt, suggesting it is functional in the mature colonocyte (Smith *et al.*, 1993). Wild-type APC forms homodimers, and mutant proteins are thought to be able to inactivate the wild-type protein in a dominant negative manner by formation of heterodimers with wild-type protein

(Su *et al.*, 1993a) APC associates with  $\beta$ -catenin (Rubinfeld *et al.*, 1993; Su *et al.*, 1993b), which is thought to transmit signals from the E-cadherin adhesion proteins to the cell's interior, suggesting a role for APC in cell adhesion and possibly intercellular communication. In cell lines, wild-type, but not mutant, APC associates with the entire microtubule skeleton when present at elevated levels (Munemitsu *et al.*, 1994; Smith *et al.*, 1994) and there is evidence that it may be important for inducing polymerisation of tubulin (Munemitsu *et al.*, 1994). However, a recent study demonstrates that endogenous APC is only found near the ends of microtubules that protrude into actively migrating membrane structures of epithelial cells, suggesting that APC might be involved in directed cell migration (Näthke *et al.*, 1996). APC has also been shown to bind EB1, although the significance of this is not clear since the function of EB1 is not yet known (Su *et al.*, 1995). Expression of APC in colorectal cancer cells lacking endogenous APC increases the number of cells undergoing apoptosis (Morin *et al.*, 1996), and it has been postulated that APC is responsible for control of tissue compartmentalism of proteins involved in proliferation, differentiation and apoptosis (Polyak *et al.*, 1996a). Finally, an increased tendency for apparently normal fibroblasts and lymphocytes from FAP patients to become tetraploid *in vitro* might suggest a role for APC in maintenance of chromosomal stability (Delhanty *et al.*, 1983).

#### ***1.3.1.1. Factors modifying the polyposis phenotype***

Patients display remarkable differences in phenotype according to the location of the mutation within the APC gene. Mutations occurring 3' to exon 9 are associated with CHRPE, whereas those occurring 5' to this boundary are not (Olschwang *et al.*, 1993). Similarly, those occurring 5' to exon 157 are associated with an attenuated disease phenotype (later onset of disease and fewer polyps) whilst those 3' to exon 168 display more aggressive disease phenotype (Spirio *et al.*, 1993), and a small central portion of the coding region is associated with profuse polyp formation (Nagase *et al.*, 1992). The observed differences in phenotype may partly be accounted for by the extent to which normal APC function is impaired by binding of a mutant form. Wild-type APC forms homodimers, but mutant APC can also bind wild-type APC, thus allowing it the potential to reduce the activity of the normal protein and act in a dominant negative fashion (Joslyn *et al.*, 1993; Su *et al.*, 1993a). Severely truncated proteins may be unable to form dimers, whereas longer proteins may more effectively sequester wild-type protein (Cunningham & Dunlop, 1994).

The polyposis phenotype can also be attenuated by several other environmental and genetic factors, which may eventually prove beneficial to the clinical management of FAP, and possibly of other APC-induced tumours. *Min* mice develop multiple bowel polyps in a similar manner to FAP patients as a result of carrying a heterozygous mutation of the APC gene (Moser *et al.*, 1990), and their phenotype is modulated by an unlinked locus known as

*mom1* (modifier of *min*) (Moser *et al.*, 1992; Dietrich *et al.*, 1993). The *mom1* locus provides a good illustration of the complexity of interaction between multiple genetic loci and diet in colorectal cancer. *Mom1* locus has been shown to be genetically identical to the gene encoding a metabolic enzyme, type II non-pancreatic phospholipase A2 (*Pla2s*) (MacPhee *et al.*, 1995). Low polyp number is associated with a null mutation in one allele of the gene. *Pla2s* probably modifies polyp number by altering the cellular microenvironment within the colonic crypts, hydrolysing phosphoglycerides to free fatty acids and lysophospholipids. It is one of the enzymes responsible for the production of arachidonic acid, which in turn is rate limiting for the production of prostaglandins. A decrease in levels of specific prostaglandins is thought to be associated with protection against tumours and also regression of polyps. However, it has been suggested that the action of *Pla2s* is complex, involving several different mechanisms, including lipid homeostasis and digestion of dietary fats, maintenance of normal bacterial flora, and elimination of aberrant crypt cells (MacPhee *et al.*, 1995).

Other genetic and epigenetic factors may also modify the polyposis phenotype. Hypomethylation induced both chemically and by crossing with mice deficient for DNA methyltransferase (*Dnmt* mice) results in drastically reduced polyp number in *min* mice (Laird *et al.*, 1995). This is suggested to be due to altered gene expression, as discussed in 1.3.10, or due to a decrease in the rate of spontaneous mutations occurring at methylated bases, decreasing the chance of knocking out the second APC allele which drives adenoma formation. *Min* mice lacking the matrix metalloproteinase (MMP) matrilysin, which is expressed in a high percentage of early human colorectal tumours, also exhibit markedly reduced polyp number and size (Wilson *et al.*, 1997). In another model, mice carrying a truncating mutation of APC have drastically reduced intestinal polyp number when Cox-2 is not expressed (Oshima *et al.*, 1996). Cox-2 encodes an isoform of cyclooxygenase (Cox), an enzyme responsible for metabolism of arachidonic acid to prostaglandins. As noted above, decreased levels of prostaglandins are associated with protection against tumour formation. Cox-2 is expressed at high levels in many colorectal tumours (Sano *et al.*, 1995), and a recent study has demonstrated a direct link between inhibition of growth of cell lines derived from colorectal cancer and selective inhibition of the Cox-2 pathway (Sheng *et al.*, 1997). Treatment of FAP patients with the NSAID sulindac, which causes regression of polyps, is associated with a decrease in levels of Cox-2 within colonic tumours (Kargman *et al.*, 1995).

### 1.3.2. The p53 gene

The product of the p53 gene is a key participant in the maintenance of genomic stability, and loss of normal p53 activity contributes to a wide variety of human cancers (Nigro *et al.*, 1989; Hollstein *et al.*, 1991; Greenblatt *et al.*, 1994). The p53 gene, located on chromosome 17p13 (Benchimol *et al.*, 1985; Isobe *et al.*, 1986; McBride *et al.*, 1986),

encodes a nuclear phosphoprotein which has the ability to bind DNA and regulate transcription of genes involved in cell cycle arrest and induction of apoptosis. In normal cells, p53 activity increases in response to events that may lead to uncontrolled cell growth, such as DNA damage, - (Gottlieb & Oren, 1996) and for this reason it has been termed the 'guardian of the genome' (Lane, 1992). p53 plays a critical role in the maintenance of genomic stability by a variety of mechanisms, including cell cycle arrest, induction of apoptosis and involvement in DNA replication and repair pathways.

The functions of p53 are complex and wide-ranging and it is not within the scope of this thesis to discuss them all in detail. An overview of its main functions, with more detailed discussion of its role in genomic instability, is presented.

#### *1.3.2.1. The role of p53 in cell cycle arrest and apoptosis*

Under genotoxic stress, such as gamma irradiation or certain chemotherapeutic drugs, different cell lineages are able to undergo p53-dependent cell-cycle arrest at the G1/S phase boundary ('G1 arrest') that is mediated by the cyclin-dependent kinase inhibitor p21 (also called WAF1 or CIP1) (Diller *et al.*, 1990; Kastan *et al.*, 1991; Mercer *et al.*, 1991; Kuerbitz *et al.*, 1992; Lane *et al.*, 1992; Lin *et al.*, 1992; El-Deiry *et al.*, 1994; Lowe *et al.*, 1993a; Di Leonardo, 1994). This arrest is thought to be necessary in order to permit repair prior to engagement of the replication complex and thus prevent the replication of mutated DNA liable to compromise genomic stability. DNA strand breaks are sufficient to trigger elevation in p53 levels and G1 arrest (Nelson & Kastan, 1994; Huang *et al.*, 1996a), and Jayaraman & Prives (1995) showed that p53 is stimulated to bind specific DNA targets by short oligonucleotides which simulate the broken DNA structures resulting from DNA damage. p53-dependent G1 arrest can occur also in the absence of DNA damage, in response to nucleotide depletion (Livingstone *et al.*, 1992; Yin *et al.*, 1992; Linke *et al.*, 1996), hypoxia and heat (Graeber *et al.*, 1994), but the G1 arrest induced by the latter two factors occurs through a combination of p53-dependent and p53-independent mechanisms. A p53-dependent G2 arrest occurs in the presence of microtubule poisons (Cross *et al.*, 1995; Dileonardo *et al.*, 1997), and after exposure to ionizing radiation, though the latter response appears to be partially p53-independent (Guillouf *et al.*, 1995; Agarwal *et al.*, 1995).

In some cell types, the induction of wild-type p53 in response to DNA damage results in cell death by apoptosis rather than cell cycle arrest (Yonish-Rouach *et al.*, 1991; Shaw *et al.*, 1992; Clarke *et al.*, 1993; Lowe *et al.*, 1993a). The mechanism of p53-mediated apoptosis is not clear; p53 is known to initiate apoptosis through up-regulation of Bax and repression of Bcl-2 (Miyashita *et al.*, 1994), though neither is absolutely required for p53-dependent apoptosis (Knudson *et al.*, 1995). p53 may also up-regulate the apoptosis



promoters KILLER/Dr5 (Wu *et al.*, 1997) and Fas/APO-1 (Owen-Schaub *et al.*, 1995), though p53-dependent activation of apoptosis can occur independently of Fas through CPP32 $\beta$  (Fuchs *et al.*, 1997).

It is not known what determines the outcome of p53 induction. It is suggested that cells can tend toward an apoptotic response if the components of the pathway of p53-induced G1 arrest are deregulated or abrogated (discussed in Gottlieb & Oren, 1996). Whilst cells respond to p53 induction following insult in a cell type-specific manner, certain cells, such as those derived from colorectal cancer, naturally undergo either growth arrest ('A-lines') or apoptosis ('D-lines') in response to the same stimulus (Lowe *et al.*, 1994; Polyak *et al.*, 1996b). p21-mediated growth arrest appears to protect A-lines from apoptosis, but fusion of both types of colorectal cancer cells indicates that the apoptotic response is dominant, suggesting the presence of a trans-acting factor which overcomes the protective effect of p21 (Polyak *et al.*, 1996b). Expression of oncogenes also influences the apoptotic response. For example inappropriate induction of c-myc in quiescent fibroblasts, though capable of driving cells into S phase despite the presence of high levels of p53, results in cell death by p53-dependent apoptosis (Hermeking & Eick, 1994). In a similar manner, suppression of transformation of E1A-expressing cells is achieved by p53-dependent apoptosis (Lowe *et al.*, 1994). p53-induced apoptosis might be a common mechanism for removal of cells that, through oncogene expression, have bypassed the G1/S checkpoint, thus preventing tumour formation. The tumour suppressor function of p53 may rely, at least in part, on its ability to induce apoptosis, so that when loss of p53 means that p53-mediated cell death no longer counterbalances proliferation, cell numbers increase (Donehower *et al.*, 1995). The appearance of aberrant p53 protein is associated with resistance to certain chemotherapeutic agents in a variety of different tumour types, including colorectal cancers (for review see Wu & El-Deiry, 1997), and this is thought to be due at least in part to resistance to apoptosis conferred by loss of normal p53 function (Lowe *et al.*, 1993b).

Very recent evidence suggests that p53 interacts with another potential tumour suppressor gene product, p33<sup>ING1</sup> (Garkavtsev *et al.*, 1998). Although little is known about this protein as yet, p33<sup>ING1</sup> is known to be a nuclear protein which physically associates with p53 and modulates the ability of p53 to act as a transcriptional activator. Neither of the two genes can cause growth inhibition when the other is suppressed, and transcription from the p21 promoter depends on expression of p33<sup>ING1</sup>. Thus, p33<sup>ING1</sup> appears to be a key component of the p53 signalling pathway important in negative regulation of cell proliferation. The determination of its importance in other p53-dependent cellular processes will be of great interest.

### 1.3.2.2 p53 and control of genomic stability

Many lines of evidence suggest that abnormalities of p53 strongly predispose to chromosomal abnormalities. Individuals with the rare inherited Li-Fraumeni syndrome carry one germ-line mutated copy of p53, and are at increased risk of a wide range of tumours, especially soft tissue sarcomas, brain tumours, osteosarcomas, breast carcinomas, leukaemias and adrenocortical carcinomas (Li and Fraumeni, 1969). Normal fibroblasts from such individuals accumulate *in vitro* genomic aberrations such as polyploidy and random chromosome losses, followed by development of dicentric chromosomes, double minutes, chromosome breaks and telomeric associations (Bischoff *et al.*, 1990) with concomitant loss of the wild-type p53 allele (Yin *et al.*, 1992). These cells also have the ability to undergo amplification of the CAD gene (encoding the trifunctional enzyme carbamoyl-P synthetase, aspartate transcarbamylase, dihydroorotase) in response to the uridine biosynthesis inhibitor PALA [N-(phosphonacetyl)-L-aspartate] (Livingstone *et al.*, 1992; Yin *et al.*, 1992). Furthermore, such cells develop changes in morphology, anchorage-independent growth and escape senescence, though they remain non-tumorigenic when explanted into nude mice (Bischoff *et al.*, 1990). This instability phenotype appears to be cell type-specific; Epstein-Barr virus (EBV)-immortalized lymphoblastoid cells from both normal and Li-Fraumeni individuals maintain functional genomic stability during long term *in vitro* culture, a feature which may contribute to selective tissue localisation of tumours in Li-Fraumeni patients (Lalle *et al.*, 1995).

Studies of murine cells deficient for p53 show a similar pattern. Mice with two defective p53 alleles are developmentally normal but succumb to tumours (predominantly lymphomas) by the age of 9-10 months (Donehower *et al.*, 1992; Harvey *et al.*, 1993a; Purdie *et al.*, 1994) whilst heterozygotes acquire tumours at a later age (Harvey *et al.*, 1993a). In culture, cells derived from such mice have very unstable karyotypes, with a strong tendency to increase their ploidy levels during growth (Harvey *et al.*, 1993b; Purdie *et al.*, 1994) and an ability to undergo gene amplification (Livingstone *et al.*, 1992; Yin *et al.*, 1992). These cells frequently undergo lack of cell partition at anaphase or unequal segregation of chromosomes due to abnormal amplification of centrosomes (Fukasawa *et al.*, 1996). The acquisition of genomic change in these cells is halted by introduction of wild-type p53 (Agapova *et al.*, 1996). Mice with p53-deficient mammary adenocarcinomas (Wnt-1 transgene/p53-null crosses) also develop tumours which frequently display aneuploidy, amplification and deletion (Donehower *et al.*, 1995). Normal murine fibroblasts, like human colon cancer cells, display an increase in the frequency of chromosome breaks and emergence of hyperdiploid cells on expression of exogenous mutant p53 (Agapova *et al.*, 1996). Deficiency of p53 does not, however, affect the rate of point mutation (Nishino *et al.*, 1995; Sands *et al.*, 1995). Finally,

in colorectal cancer, a strong significant correlation is seen between p53 mutation and aneuploidy, including allelic losses of chromosome regions 17p, 18q and 5q and hypotetraploid DNA index (Bell *et al.*, 1993; Meling *et al.*, 1993; Hamelin *et al.*, 1994). Moreover, abnormal p53 function arises as an event preceding, and perhaps facilitating, clonal divergence of carcinomas in sporadic bowel lesions (Carder *et al.*, 1993 & 1995).

Gottlieb & Oren (1996) propose that p53 maintains genomic stability, firstly, through its role in G1 arrest, preventing gross chromosomal abnormalities such as translocation, deletion or amplification by inhibition of replication of cells in which DNA is damaged, and, secondly, through a G2 'spindle-surveillance' checkpoint, which prevents the formation of aneuploid and polyploid cells. As a result of loss of normal p53 function, loss of cell-cycle control and accompanying genomic instability favours a rapid accumulation of mutations necessary for tumour development. As a further control, p53 regulates induction of apoptosis, which is important in the elimination of damaged cells.

#### *1.3.2.3. Mutation of p53 in colorectal cancer*

Loss of normal p53 function is the most common genetic lesion in sporadic colorectal cancer, occurring in about 75% of cases (Baker *et al.*, 1990; Cunningham *et al.*, 1992; Kaklamanis *et al.*, 1993), frequently through mutation of the gene. Inactivation of p53 in sporadic cancer often involves both alleles; allelic deletions occur in more than 75% of carcinomas (Vogelstein *et al.*, 1988; Cunningham *et al.*, 1992), accompanied by mutations of the second allele in 86% of cases (Baker *et al.*, 1990). p53 mutations arising in sporadic colorectal carcinoma are predominantly missense, and frequently result in the nuclear accumulation of abnormal protein with increased half-life, possibly through altered conformation, oligomerization or interactions with other proteins such as certain heat shock proteins (Finlay *et al.*, 1988; Sturzbecher *et al.*, 1988; Kraiss *et al.*, 1991), allowing detection by immunocytochemical methods (Levine, 1991). In some cells containing both mutant and wild-type p53, the mutant form appears to stabilize the otherwise rapidly-degraded wild-type protein through the formation of oligomers, possibly by altering the conformation of the wild-type protein and thus behaving in a dominant negative fashion (Eliyahu *et al.*, 1988; Milner & Medcalf, 1991). Mutant p53 can also, under some circumstances, exhibit gain of function (Dittmer *et al.*, 1993). The cellular environment is important in maintenance of normal p53 function, since this can be abrogated by indirect interaction with viral proteins such as SV40 largeT antigen, HPV E6 and adenovirus E1b, and by cellular factors such as the proto-oncogene Mdm2 (discussed in Blagosklonny, 1997). In some human sarcomas, overexpressed Mdm-2 binds to and stabilises p53, resulting in functional inactivation (Oliner *et al.*, 1992), and may be involved in degradation of p53 (Haupt *et al.*, 1997).



Mutations of the p53 gene, deletions of chromosome 17p and nuclear stabilisation of the p53 protein occur rarely in adenomas (Vogelstein *et al.*, 1988; van den Berg *et al.*, 1989; Baker *et al.*, 1990; Fearon and Vogelstein, 1990; Rodrigues *et al.*, 1990; Campo *et al.*, 1991; Shirasawa *et al.*, 1991; Kikuchi-Yanoshita *et al.*, 1992; Starzynska *et al.*, 1992; Kaklamani *et al.*, 1993; Ried *et al.*, 1996), indicating that loss of p53 function is associated with the transition from adenoma to carcinoma. p53 abnormality or loss occurs more frequently in distal colorectal cancers (Scott *et al.*, 1991; Bell *et al.*, 1993; Laurent-Puig *et al.*, 1992; Meling *et al.*, 1993; Hamelin *et al.*, 1994), and there is some evidence that p53 abnormality is an independent predictor of poor prognosis in colorectal cancer (Sun *et al.*, 1992; Stargynza *et al.*, 1992; Hamelin *et al.*, 1994), although others find that it is not (Bell *et al.*, 1993).

Given the apparent importance of abrogation of G1 arrest through loss of p53 function in colorectal cancer, tumours containing normal p53 gene product might be expected to harbour defects in other components of this pathway, such as p21. However, somatic mutations of p21 have been searched for in a series of colorectal lesions but no mutations have been found (Li *et al.*, 1995). Disruption of p21 protein distribution has been shown to occur in dysplastic ACFs (Polyak *et al.*, 1996a), in which normal tissue compartmentalism of the protein, in the upper non-proliferating third of the crypt, is lost.

#### ***1.3.2.4. The role of p53 in DNA replication and repair***

Evidence that p53 is directly involved in nucleotide excision repair (NER) comes from the observation that the DNA repair rate is reduced in cells from individuals with Li-Fraumeni syndrome and in colon cancer cells with abrogated p53 function (Ford *et al.*, 1995; Smith *et al.*, 1995; Wang *et al.*, 1995). p53 is probably involved in control of NER and DNA replication by both transcriptional and non-transcriptional means. p53 interacts with several cellular components thought to be involved in replication and repair, including the single strand binding protein RPA, XPB, XPD and CSB (for review see Gottlieb & Oren, 1996). In addition, it interacts with the DNA damage-induced GADD45 protein (Kastan *et al.*, 1992), and through this activity may indirectly regulate levels of proliferating cell nuclear antigen (PCNA) required for DNA synthesis in both replication and repair. The regulation of p21 by p53 may also influence levels of PCNA (Mercer *et al.*, 1991), since p21 has been shown to modulate PCNA activity (Li *et al.*, 1994). The inhibitory effect of p53 on the replication machinery may contribute to its ability to block the cell cycle at G1.

There is some evidence that the pathways of detecting and responding to DNA damage may be interconnected. A recent report demonstrates the ability of p53 to bind the promoter region of hMSH2, thus implicating p53 in the regulation of hMSH2 and possibly implying a role for it in mismatch repair (Scherer *et al.*, 1996), and there is evidence that p53 plays a direct role in recognition of insertion/deletion mismatches (Lee *et al.*, 1995). This has

important implications for the study of p53 and mismatch repair defects in colorectal cancer, since in general tumours with defects in p53 tend not to have deficiencies in the MMR pathway (Ionov *et al.*, 1993; Kim *et al.*, 1994a; Cottu *et al.*, 1997; Remvikos *et al.*, 1997). This is discussed further later in this thesis.

In summary, though p53 may maintain stability of the genome by a variety of different methods which are not yet fully understood, abnormalities of p53 have been unequivocally linked with chromosomal abnormalities in a number of different systems. Furthermore, there is strong evidence that loss of normal p53 leads to aneuploidy in colorectal cancer. It is on the basis of this evidence that the relationship between p53 abnormalities and chromosomal anomalies were examined in this study.

### 1.3.3. Genes located on chromosome 18q

Loss of chromosome 18q occurs in about 70% of colorectal cancers, and is thus thought to be a very important step in tumour progression (Vogelstein *et al.*, 1988). Loss of this region characterises the progression from early to late adenoma, and appears to be almost universal in colorectal metastases (Ookawa *et al.*, 1993; Frank *et al.*, 1997). Chromosome 18q status has strong prognostic value in Dukes' stage B cancers, with loss predicting poor survival (Jen *et al.*, 1994b). At least three putative tumour suppressor genes are located in this region.

A gene in the commonly deleted region of 18q, DCC (Deleted in Colorectal Cancer), was identified and found to exhibit deletion, somatic mutation and absent or greatly reduced expression in most colorectal cancers (Vogelstein *et al.*, 1988; Fearon *et al.*, 1990; Itoh *et al.*, 1993; Cho *et al.*, 1994). DCC has been shown to be involved in cell adhesion, and expression of antisense DCC transforms Rat-1 fibroblasts (Narayanan *et al.*, 1992). In flies and vertebrates, DCC and related proteins are expressed in gut epithelia, and are readily detectable on the mucin-secreting goblet cells (Hedrick *et al.*, 1994; Kolodziej *et al.*, 1996). Colorectal cancer cells lacking DCC appear to be deficient in mucin staining, possibly because either the goblet cells do not contribute to these cancers or because DCC is required for differentiation to mucin-producing cells, whereas mucinous carcinomas almost always retain DCC (Hedrick *et al.*, 1994). The gene encodes a netrin-1 receptor, and is probably involved in mediating axon guidance (Keino-Masu *et al.*, 1996). Its role in neuronal morphogenesis suggests that, in the gut, DCC may help to maintain tissue integrity or cell morphology, and loss may contribute to metastasis (Kolodziej, 1997).

A subset of colorectal cancers demonstrate deletion and mutation of a second gene located within 1Mb of DCC, DPC4 (Takagi *et al.*, 1996; Thiagalingam *et al.*, 1996). DPC4 shows sequence homology to the drosophila gene *Mad*, which is thought to transduce signals from transforming growth factor  $\beta$  (TGF $\beta$ ) family members. A third closely-linked *Mad*-

related gene, *JV18*, is shown to be mutated in a proportion of colorectal cancers (Riggins *et al.*, 1996) and a fourth, *MADR2* is also mutated in a subset of colorectal cancers (Eppert *et al.*, 1996). Mutation of another component of the TGF $\beta$  growth-control pathway, the second subunit of the TGF $\beta$  receptor (TGF $\beta$ RII), is almost ubiquitous in a subset of colorectal tumours with microsatellite instability (Markowitz *et al.*, 1995) and it is suggested that mutation of genes encoding components of the TGF $\beta$  signal transduction pathway may be one method by which colorectal cancers without microsatellite instability become insensitive to the growth-inhibiting properties of TGF $\beta$ .

Thymidylate synthase (TS), a target enzyme for several chemotherapeutic drugs including 5-fluorouracil (5-FU), is also encoded by a gene on chromosome 18q. TS expression is reduced in a large proportion of colorectal cancers (Lasserre *et al.*, 1994), which might be expected to exhibit reduced drug sensitivity. However, whether a correlation exists between TS expression and response to 5-FU remains controversial (Johnston *et al.*, 1995; Findlay *et al.*, 1997).

The Bcl-2 gene, also located on chromosome 18q, is discussed below.

#### 1.3.4. The ras genes

Although loss of function of tumour suppressor genes occurs frequently in colorectal cancer, activation of proto-oncogenes is generally less prevalent. However, mutation of the Kirsten ras (Ki-ras) oncogene is often acquired during colorectal tumour development. Approximately 50% of colorectal carcinomas and adenomas >1cm in diameter harbour Ki-ras mutations, predominantly in codons 12 and 13 (Bos *et al.*, 1987; Forrester *et al.*, 1987; Vogelstein *et al.*, 1988).

The ras family of genes are cytoplasmic proto-oncogenes involved in signal transduction (Barbacid *et al.*, 1987) which, in conjunction with other oncogenes, are capable of transforming cells in culture (Ruley *et al.*, 1983; Newbold *et al.*, 1983). Furthermore, disruption of mutant Ki-ras in cell lines derived from colorectal cancer has been shown to result in altered cell morphology, loss of anchorage-independent growth, slower growth, a reduction in tumorigenic potential, and concomitant reduction in expression of the c-myc oncogene, suggesting a key role for Ki-ras in maintenance of the cancer phenotype in these tumour lines (Shirasawa *et al.*, 1993).

#### 1.3.5. Bcl-2 and Bax

Abrogation of proteins directly involved in the control of apoptosis could clearly be of importance in maintaining normal cell turnover and preventing tumour growth. Two such proteins, Bcl-2 and Bax, have been examined in colorectal cancer. Bcl-2 which encodes a mitochondrial membrane protein, the expression of which inhibits programmed cell death (for review see Reed, 1994). Detectable Bcl-2 transcript is completely lacking in over half of all

sporadic colorectal cancers, and this is associated with poor prognosis (Öfner *et al.*, 1995). The role of Bcl-2 in carcinogenesis is not clear; lack of transcript might be expected to facilitate apoptosis, and therefore cause a reduction in tumour growth. However, this paradoxical inhibition of tumour growth may be explained by the location of Bcl-2 in a commonly deleted region of chromosome 18q, where Bcl-2 is deleted as a 'passenger' along with other genes of importance in tumour growth (Pietenpol *et al.*, 1994). Expression of Bax, which promotes the apoptotic process, is also lacking in a over 50% of colorectal cancers with microsatellite instability, due to mutations in a polynucleotide tract within the coding region of the gene (Rampino *et al.*, 1997).

#### 1.3.6. The c-myc gene

Expression of the oncogene c-myc is frequently increased in sporadic colorectal cancer (Gallick *et al.*, 1985; Sikora *et al.*, 1987), sometimes due to amplification of the gene (Kafatos *et al.*, 1985). The c-myc gene encodes a nuclear protein with DNA binding capacity which is capable of inducing apoptosis at all points throughout the cell cycle (Evan *et al.*, 1992) and thought to be essential for cell proliferation (Heikkila *et al.*, 1987). Elevated levels of c-myc product are not seen in tumours with distant metastases, or in the metastases themselves, indicating its involvement at an earlier stage of development (Gallick *et al.*, 1985). It is not yet known whether expression of c-myc is directly related to tumour initiation or progression.

#### 1.3.7. Other putative tumour suppressor genes in colorectal cancer

Frequent loss of heterozygosity indicates that regions of the genome harbour as yet unidentified tumour suppressor genes important in colorectal cancer. Partial deletion of chromosome 1p occurs in up to half of all colorectal cancers (Reichmann *et al.*, 1984; Leister *et al.*, 1990; Muleris *et al.*, 1990; Couturier-Turpin *et al.*, 1992; Bardi *et al.*, 1993a & 1995; Bomme *et al.*, 1994; di Vinci *et al.*, 1996) and is associated with poor prognosis (Gerdes *et al.*, 1995). Some studies find that it also occurs in a high proportion of adenomas, with frequency of loss lowest in tumours with low levels of dysplasia and highest in early foci of cancer (Bardi *et al.*, 1993a; Bomme *et al.*, 1994; DiVinci *et al.*, 1996); other studies find 1p loss to be a later event (Reichmann *et al.*, 1984; Leister *et al.*, 1990; Longy *et al.*, 1993). Detailed deletion mapping suggests that at least three separate loci may be involved (Leister *et al.*, 1990; Praml *et al.*, 1995a), though no tumour suppressor genes have yet been unequivocally identified. Introduction of 1p36 into colon cancer cells suppresses tumorigenicity (Tanaka *et al.*, 1993), and this region contains a candidate tumour suppressor, p73, which encodes a protein with homology to p53 that can activate p53 target genes and interact with p53 (Kaghad *et al.*, 1997). Screening of the remaining allele in neuroblastoma, where this region of 1p is also frequently deleted, does not demonstrate mutations, but this

gene has yet to be examined in colorectal cancer. In mice, Pla2s (Type II non-pancreatic phospholipase A2), the gene thought to be the modifier of the *min* phenotype, *Mom-1*, is located on the region of the mouse genome syntenic to human 1p35-36 (Praml *et al.*, 1995b), a region often involved in deletion in colorectal cancer (MacPhee *et al.*, 1995). However, since the mode of action of Pla2s appears to be non-cell-autonomous, with the product being most active in the colonic lumen rather than within the cell, it is difficult to see how loss of this gene in a cell would be selected for in neoplastic progression. Indeed, somatic mutations of this locus in colorectal tumours in which one allele is deleted are extremely rare (Riggins *et al.*, 1995).

Loss of material on chromosome 8p also occurs in about half of all sporadic colorectal carcinomas (Cunningham *et al.*, 1993) and is associated with the change from adenoma to carcinoma (Cunningham *et al.*, 1994). There is strong evidence to suggest that this region of the genome harbours at least two colorectal tumour suppressor genes (Cunningham *et al.*, 1993; Fujiwara *et al.*, 1993; Yaremko *et al.*, 1994; Farrington *et al.*, 1996), though no such gene has yet been convincingly identified. A recently-identified candidate gene is KILLER/DR5, a tumour necrosis factor (TNF)-receptor related protein involved in p53-dependent apoptosis. Partial gene screening of the remaining allele in colorectal cancers has not yet identified mutations (Wu *et al.*, 1997).

Loss of heterozygosity studies in which the tumour suppressor gene APC was identified initially identified another gene, MCC (mutated in colorectal cancer), located within 100kb of APC, as being frequently lost in colorectal cancer (Solomon *et al.*, 1987; Vogelstein *et al.*, 1988; Ashton-Rickardt *et al.*, 1991; Miki *et al.*, 1991). The gene product of MCC shows sequence homology to a murine G protein receptor, important in signal transduction. Mutations in the gene were detected in several sporadic cancers, leading to speculation that it may play an important role in colorectal cancer development (Kinzler *et al.*, 1991b). However, no further mutations have been reported and the role of MCC in colorectal carcinogenesis appears to be very limited (Curtis *et al.*, 1994).

There may be a role for retinoblastoma (Rb) in colorectal cancer, but, at least in earlier stage cancers, it does not appear to function as a tumour suppressor gene (Meling *et al.*, 1991). Rather, it undergoes low-level amplification in almost 30% of cancers, though this tends to happen in aneuploid cancers and may therefore be coincidental to whole chromosome 13 gain. It may, however, play a role in suppression of metastasis (Ookawa *et al.*, 1993; Young *et al.*, 1993).

#### 1.3.8. Genetic lesions and metastasis

Little is known about the genetic requirements for metastasis. Loss of heterozygosity of the gene nm-23, located on chromosome 17q21, is associated with metastatic progression



(Cohn *et al.*, 1991; Leone *et al.*, 1991; Wang *et al.*, 1993), although this is not found in all studies (Haut *et al.*, 1991). Loss of the p53 and DCC genes appear to be essential to metastasis (Ookawa *et al.*, 1993; Frank *et al.*, 1997). In addition, loss of regions of 13q (including the Rb locus) and 14q occur more frequently in later stage carcinomas and liver metastases when compared to the corresponding primary tumours (Ookawa *et al.*, 1993; Young *et al.*, 1993a). Motility-related protein (MRP/CD9), a gene implicated in cancer cell motility and metastasis, is down-regulated in metastatic lesions compared with primary tumours (Cajot *et al.*, 1997).

#### 1.3.9. Killing of host immune cells through FasL

Intriguing new findings indicate novel mechanisms by which cancer cells survive and proliferate. One of the ways in which cancer cells do this may be through the disabling of host immune response. *In vitro* experiments suggest that colon cancer cells may be involved in the active killing of immune cells. Cell line SW620 (derived from a lymph node metastasis) expresses functional Fas ligand (FasL), the triggering agent of Fas receptor (FasR)-mediated apoptosis within the immune system and a key molecule in normal immune development. SW620 is capable of killing Jurkat T cells in a Fas-mediated manner but, despite also expressing FasR itself, is resistant to being killed in a Fas-mediated manner (O'Connell *et al.*, 1996).

#### 1.3.10. DNA Methylation

Extensive hypomethylation is evident within the genomes of colonic neoplasms, occurring even in very small benign adenomas (Goelz *et al.*, 1985a). DNA is methylated by a covalent modification of cytosine residues by DNA methyl transferase, a modification which affects gene expression (for refs see Goelz *et al.*, 1985a). Hypomethylation does not appear to be a feature of hyperplastic epithelium, and is therefore thought to be an early event in tumour development, preceding malignancy. In colorectal cancer, reduction in methylation occurs selectively, with some genes consistently hypomethylated and others variable. Thus, whilst hypomethylation would allow increased expression of proto-oncogenes, specific areas of hypermethylation can occur and expression of tumour suppressors could be reduced in this way (deBustros *et al.*, 1988; Silverman *et al.*, 1989). This may even be driven by hypomethylation of genes such as DNA methyltransferase, although the mechanism of gene selection is unclear (Laird *et al.*, 1995).

Reduced methylation may contribute to genomic instability. Strand breaks are associated with hypomethylation, probably because a reduction in methylation affects chromatin structure, allowing increased access of changed regions to DNA damaging agents such as nucleases (Lewis & Bird, 1991). In addition, it reduces binding sites for methyl-specific proteins, making some regions more accessible to oxidant- and/or enzyme-induced

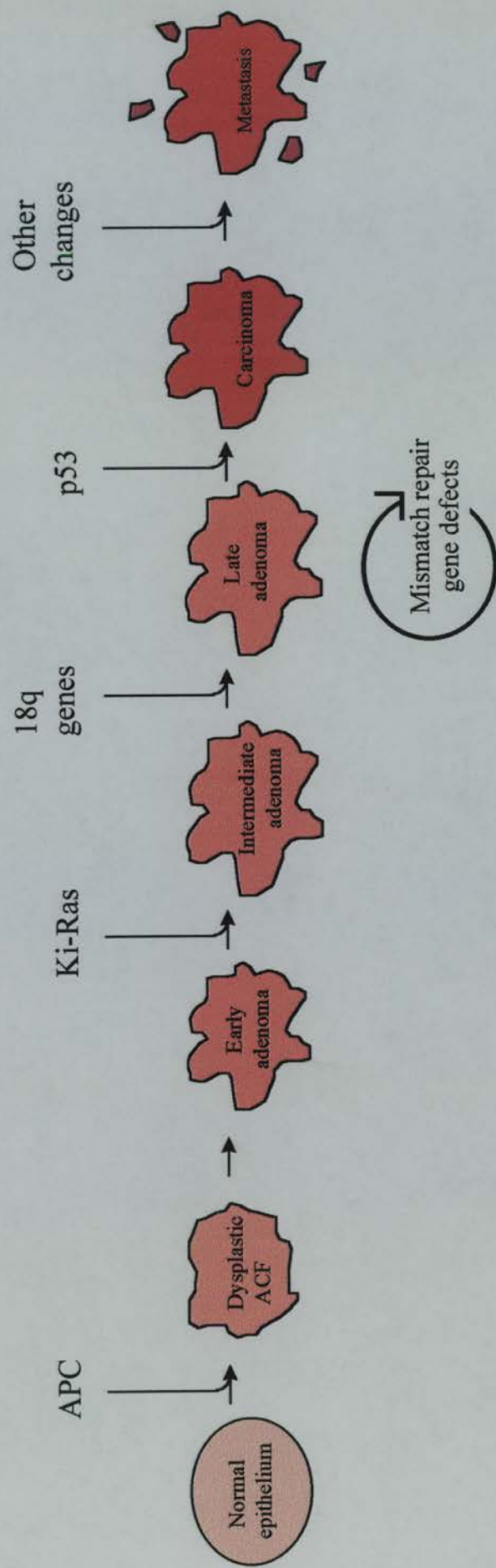
strand breaks. Loss of chromosome condensation in this way may also lead to chromosome non-disjunction (Schmid *et al.*, 1984). Thus, hypomethylation may facilitate whole or partial chromosome loss or gain and chromosomal rearrangement.

## **Figure 1**

### **Genetic changes associated with the development of colorectal cancer**

This figure, based on a model proposed by Fearon and Vogelstein, (1996), shows the temporal relationship between critical genetic events thought to be involved in development of most colorectal cancers. APC mutations are thought to initiate tumour formation in both sporadic and inherited colorectal cancer, and are present in the germ-line of FAP patients. Neoplasia progresses as a result of mutations in Ki-ras, one or more tumour suppressor genes located on chromosome 18q, which include DCC, DPC4 and JV18, and p53. Mismatch repair gene mutations, a feature of almost all HNPCC cancers and a proportion of sporadic cancers, may speed up the tumorigenic process by increasing the rate of mutation in these or other genes, but also predispose to mutations in other genes where 'target' repetitive sequences are present, such as in the TGF $\beta$  receptor subunit II (TGF $\beta$ RII), Bax and hMSH6. The genetic events associated with metastasis are poorly understood, but may involve loss of function of nm-23, p53, DCC and MRP/CD9.





### 1.3.11. Microsatellite instability and mismatch repair defects in colorectal cancer

Analysis of Hereditary Non-Polyposis Colorectal Cancer (HNPCC) has shown underlying defects of the process of mismatch repair to be fundamental to development of cancer in patients with this disease. This has laid the basis for a deeper understanding of genetic instability in sporadic colorectal cancer.

The genetics of HNPCC and the mismatch repair pathway are discussed briefly below.

#### ***1.3.11.1. The genetic basis of microsatellite instability in HNPCC***

HNPCC is a dominantly-inherited disease characterised by predisposition to early-onset colorectal cancer, often with familial clustering of endometrial and ovarian cancer and sometimes stomach, small intestine, hepatobiliary, kidney and ureter cancer. It is thought to account for up to 5% of all colorectal cancers, and could affect as many as one in 200 individuals (Leach *et al.*, 1993). HNPCC kindreds are defined by the Amsterdam Criteria, which are: (i) at least three relatives with histologically verified colorectal cancer, one of them a first degree relative of the other two, (ii) at least two successive generations are affected, and (iii) cancer onset is under 50 years of age in one of the affected persons (Vasen *et al.*, 1994).

Cancers from up to 86% of HNPCC patients exhibit microsatellite instability (MSI), a term which describes the acquisition of multiple new alleles at microsatellite sequences throughout the genome (Aaltonen *et al.*, 1993; Ionov *et al.*, 1993; Wu *et al.*, 1994). Cells in which this phenomenon is present lack efficient repair of small insertions/deletions and point mutations which occur during replication, and have been designated replication error positive (RER+) when instability is detectable at two or more loci (Aaltonen *et al.*, 1993). Most individuals with HNPCC carry germ-line mutations of components of the mismatch repair pathway, principally hMSH2, which linkage analysis and mutation screening suggest accounts for about 50% of all families (Peltomaki *et al.*, 1993; Liu *et al.*, 1994; Nystrom-Lahti *et al.*, 1994; Wijnen *et al.*, 1995; Liu *et al.*, 1996a). A further 40% of families harbour germ-line mutations in the mismatch repair gene hMLH1, whilst hPMS1, hPMS2, hMSH3 and hMSH6 are thought to account for the remaining 10% (Bronner *et al.*, 1994; Nicolaides *et al.*, 1994; Papadopoulos *et al.*, 1994; Tannergard *et al.*, 1994; Liu *et al.*, 1996a; Akiyama *et al.*, 1997; Miyaki *et al.*, 1997). Interestingly, families in which colorectal cancer is common but where the criteria for HNPCC are not met harbour very few mismatch repair gene mutations (Wijnen *et al.*, 1997).

The human mismatch repair process is still poorly understood, but more is known about the homologous processes in bacterial and yeast systems. These are discussed below.

### 1.3.11.2. Mismatch repair

Mismatch repair is critical for correction of mutations arising through errors of DNA replication or some types of chemical damage. It has also been shown to play a role in the processing of recombination intermediates (Rayssiguier *et al.*, 1989; Baker *et al.*, 1995; Selva *et al.*, 1995) and in control of a cell cycle checkpoint, triggering G2 cell cycle arrest (Hawn *et al.*, 1995). Components of the mismatch repair system, MutS and MutL, have been implicated in transcription-coupled nucleotide excision repair (Mellon *et al.*, 1996). These elements are also involved in recognition of neurodegenerative disease-associated trinucleotide repeats (Pearson *et al.*, 1997), though triplet repeats in the myotonic dystrophy and fragile X locus are stable in mismatch repair-deficient cells (Kramer *et al.*, 1996). Cells in which effective mismatch repair is lacking possess a massively increased mutation rate referred to as the 'mutator phenotype'.

#### *MutHLS-type mismatch repair in E.coli and S.cerevisiae*

Understanding the processes of mismatch repair in bacterial and yeast systems has provided a basis on which to begin studying the repair process in man. The major mechanism of mismatch repair in *E.coli* is the MutHLS pathway, summarised in Figure 2a. Mismatch recognition, DNA binding, determination of strand-specificity and nicking of DNA involves three key components, MutS, MutL and MutH, whilst excision and resynthesis involves a number of other gene products. A similar mismatch repair system involving components homologous to those of the bacterial MutHLS pathway has been characterised in *S.cerevisiae* (Reviewed in Kolodner, 1996). This process is described in Figure 2b.

*S.cerevisiae* contains six proteins homologous to bacterial MutS, MSH1-6 (for MutS Homologue), and employs three of these homologues, MSH2, MSH3 and MSH6 directly in the DNA binding component of its mismatch repair pathway. MSH2 recognises and repairs both insertion/deletion and single base mispairs in conjunction with MSH6 and MSH3, although the functions of MSH3 and MSH6 are partially redundant (Acharya *et al.*, 1996; Marsischky *et al.*, 1996). The precise function of MSH4 and MSH5 is unclear, although MSH4 mutants, whilst not deficient for mismatch repair, undergo non-disjunction of homologous chromosomes at meiosis I (Ross-Macdonald *et al.*, 1994). *S.cerevisiae* has at least two homologues of MutL, MLH1 (MutL Homologue) and PMS1 (Post-Meiotic Segregation) (Reenan *et al.*, 1992 a&b), though no homologue of MutH has yet been identified. Mutations of *MSH2*, *MSH3*, *MSH6*, *PMS1* and *MLH1* and the endo/exonuclease *RTH1* all cause widespread repetitive DNA tract destabilisation similar to that observed in human cancer (Strand *et al.*, 1994 & 1995; Johnson *et al.*, 1996a; Marsischky *et al.*, 1996; reviewed in Kolodner *et al.*, 1996). Furthermore, gene conversion events are common in *S.cerevisiae* mutants lacking *PMS1*, *MSH2* and *MLH1*, suggesting a role for these components

in regulation of recombination (reviewed in Kolodner, 1996). It is not known how daughter strand recognition is accomplished in *S.cerevisiae* mismatch repair, since hemimethylation is not apparent.

#### *Mismatch repair in man*

*In vitro* studies of cells derived from human colorectal cancers with replication errors demonstrate the presence of different complementation groups and the existence of trans-acting factors with the ability to correct defective repair (Umar et al., 1994; Boyer et al., 1995). Current knowledge indicates that human cells undergo repair of base mismatches and small insertion/deletion mutations by a process similar to that of *S.cerevisiae* (Figure 2c). hMSH2, a 97kDa protein homologous to yeast MSH2 and bacterial MutS (Leach et al., 1993; Fishel et al., 1993), is capable of independently binding mismatched nucleotides (Fishel et al., 1994) and is thought to be an absolute requirement for mismatch repair; absence of hMSH2 results in an extreme mutator phenotype with numerous mutations in repeat units and increased rate of point mutation (Bhattacharyya et al., 1994; Shibata et al., 1994; Boyer et al., 1995). Generation of antibodies against hMSH2 has shown it to be an exclusively nuclear protein highly expressed in the testis, thymus and proliferative cells of the gut, but also expressed at lower levels in skin, heart, lung, skeletal muscle, smooth muscle, thyroid, liver and the germinal centres of lymphoid follicles (Wilson et al., 1995; Leach et al., 1996). hMSH2 acts in conjunction with hMSH6 (also termed GTBP, or p160) to form a heterodimer termed hMutS $\alpha$  (Palombo et al., 1995; Papadopoulos et al., 1995). hMSH6 appears to be preferentially involved in repair of base mismatches rather than frameshift mutations, since homozygous mutation results in a rate of point mutation rate 350-450-fold higher than mismatch repair-proficient cell lines [a rate comparable with that of other mismatch repair-deficient lines] (Bhattacharyya et al., 1994; Glaab et al., 1997) and frequent reductions in length of mononucleotide tracts (Drummond et al., 1995), but few microsatellite shifts (Drummond et al., 1995). A further MSH2 homologue, hMSH3 (the human homologue of yeast MSH3) appears to play a limited role in repair of some base/base mismatches and may be more important in repair of insertion/deletion mutations, though its precise role in the latter is still unclear (Risinger et al., 1996). As in yeast, MSH3 and MSH6 are partially redundant in mismatch repair (Risinger et al., 1996).

There are three human homologues of bacterial MutL, hMLH1 (homologous to yeast MLH1), hPMS2 (homologous to yeast PMS1) and hPMS1. hMLH1 is thought to act in a similar manner to its bacterial homologue MutL, facilitating the formation of the final effector complex. Hence, although mismatch binding is normal in cells lacking hMLH1 (Branch et al., 1995), such cells exhibit a similar phenotype to those deficient for hMSH2, displaying an extensive mutator phenotype with shifts in di-, tri- and tetranucleotide repeats and high

numbers of point mutations (Bhattacharya *et al.*, 1994; Parsons *et al.*, 1993; Umar *et al.*, 1994). Cell lines lacking hPMS2 display a higher rate of point mutation than those with defective hMLH1 (Glaab *et al.*, 1997) and lines lacking both hMLH1 and hPMS2 display a very high mutation rate (750-fold higher than a mismatch repair proficient control and 50-fold higher than that in hMLH1-deficient cells). hPMS1 has been implicated in mismatch repair because of the identification of a germ-line mutation in a patient with HNPCC (Nicolaidis *et al.*, 1994), but its biochemical role is not yet understood. Many aspects of mismatch repair in human cells, such as the method of strand-specific recognition and elements downstream of hMSH2/hMSH6 in the repair pathway, are still unknown.

#### *Other mechanisms of mismatch repair*

In addition to MutHLS-type repair, *E. coli* is equipped with base-specific mismatch repair mechanisms, the very short patch (VSP) repair pathway and the MutY-dependent pathway (Reviewed in Kolodner, 1995). VSP repair primarily corrects G-T mispairs to G-C, (but also repairs G-U at lower efficiency) and requires MutS, MutL, Vsr, DNA polymerase I and ligase. The MutY-dependent repair pathway is one of three systems that can repair oxidative damage to guanine, also correcting A-G and A-C mispairs to C-G and G-C at a lower rate, and involves MutY, MutT, MutM (Fpg), DNA polymerase I and ligase. It is likely that base-specific mismatch repair similar to that in *E. coli* exists in eukaryotic cells; the fact that eukaryotes preferentially repair G-T mismatches to G-C implies that they have a pathway that plays the same role as *E. coli* VSP repair, although the mechanism is likely to be different. Similarly, the existence of a human MutT homologue implies that a MutY-like pathway exists (reviewed in Kolodner, 1996). The existence of these pathways in human cells has not been established and these mechanisms will not be discussed further.



## Figure 2

### *MutHLS*-type mismatch repair in *E.coli*, *S.cerevisiae* and man.

#### A. Mismatch repair in *E.coli*

This diagram (based on Kolodner, 1996) illustrates the action of the *E.coli* MutHLS mismatch repair system on a mispair at a replication fork. Repair is initiated when a base mismatch incorporated by DNA polymerase escapes the polymerase's proofreading ability. A protein encoded by the gene *MutS* binds the mismatch (Su & Modrich, 1986) and is bound by a homodimer of the *MutL* product (Grilley *et al.*, 1989). *MutL* does not become part of the final effector complex, but acts to facilitate the formation of a stable structure between *MutS* and a third protein, *MutH*, which binds a hemimethylated GATC site either upstream or downstream of the mismatch. Thus, a stable complex forms with several thousand nucleotides of DNA containing the mismatch looped out. Daughter strand recognition occurs by virtue of the hemimethylated state of newly-synthesised DNA; *MutH* nicks the unmethylated strand and excision and repair take place with the aid of DNA helicase II (*uvrD* gene product), DNA single-stranded binding protein, DNA polymerase III holoenzyme, DNA ligase and one of the single-stranded DNA exonucleases (Reviewed in Modrich, 1991 and Kolodner, 1996). Repair can occur in either direction from the mismatch, and the molecular requirements are much the same (Cooper *et al.*, 1993).

#### B. Mismatch repair in *S.cerevisiae*

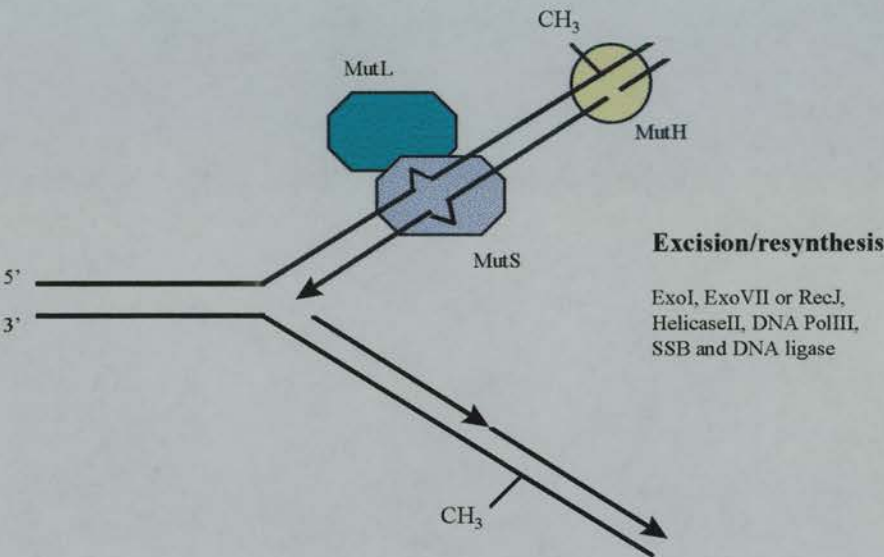
Shown here is an illustration of the mismatch repair pathway in *S.cerevisiae* (from Marsischky *et al.*, 1996). Repair of single base substitution mispairs is thought to occur through binding of mismatched DNA by the *MutS* homologue MSH2 in complex with MSH6, whilst either MSH6 or MSH3 can interact with MSH2 in the repair of insertion/deletion mispairs. Two *MutL* homologues, MLH1 and PMS1 form a heterodimer and complex with MSH2. The downstream elements of this process are poorly understood, but are thought to involve exonucleases, helicases and enzymes required for DNA synthesis.

#### C. Mismatch repair in man

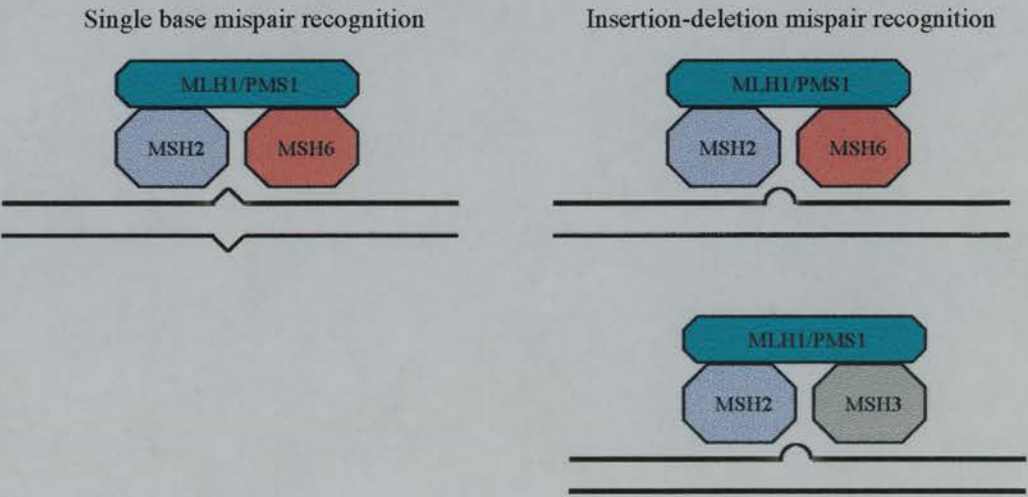
hMSH2, a human homologue of MSH2, binds single base mismatches in complex with hMSH6. A further MSH2 homologue, hMSH3, is probably involved in repair of insertion/deletion mutations, and may play a limited role in repair of some single base mismatches. hMLH1 and hPMS2 facilitate the formation of the final repair complex. Elements downstream of the MSH2 and MLH1 homologues are still unknown.



A. MutHLS mismatch repair in *E.coli*

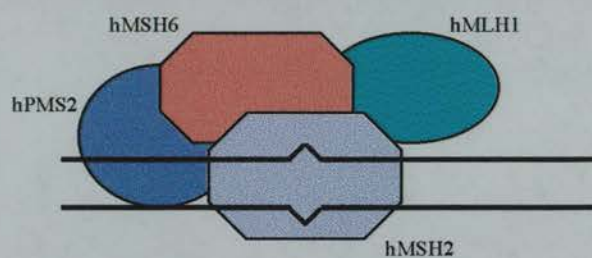


B. Mismatch repair in *S.cerevisiae*

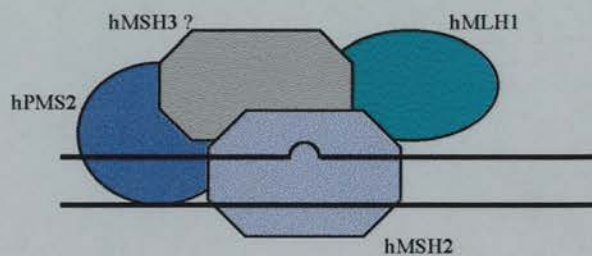


### C. Mismatch repair in man

#### Single base mispair recognition



#### Insertion-deletion mispair recognition



#### ***1.3.11.3. Mismatch repair gene products in human tumours***

In human tumours, the mismatch repair genes usually behave as classical tumour suppressor genes in their requirement for homozygous loss of function, with almost all RER+ tumour cell lines exhibiting complete lack of normal product from one mismatch repair gene (Boyer *et al.*, 1995). However, a heterozygous repair effect has been demonstrated in some RER+ cells *in vitro* (Parsons *et al.*, 1993). Unlike other tumour suppressor genes, loss of heterozygosity is not a common mechanism of gene deletion in sporadic colorectal cancer (Aaltonen *et al.*, 1993; Hemminki *et al.*, 1994), although loss of hMLH1 does sometimes occur (Tomlinson *et al.*, 1996). Expression of hMSH2 in the large intestine is normally restricted to the epithelium in the lower half of the crypts of Lieberkühn where cell proliferation occurs. This localisation is lost in at least some colonic neoplasms, which show intense staining throughout the crypt, though the authors do not state the mismatch repair status of the tumours studied (Leach *et al.*, 1996). Dysplastic crypts also lose compartmentalism of Ki67, a marker of cell proliferation, which is normally localised in the lower third of the crypt, and p21, normally in the upper non-proliferating third of the crypt. It is suggested that APC, also localised in the upper part of the crypt and the only known early genetic lesion in colorectal cancer, may control the compartmentalism of hMSH2, p21 and Ki67 within the colorectal epithelium (Polyak *et al.*, 1996a).

An intriguing function of mismatch repair *in vivo* is its apparent association with control of DNA methylation. Human cancer cells with functional mismatch repair are methylation-proficient, and thus have the ability to transcriptionally deactivate genes, whereas mismatch repair-deficient cells are methylation-deficient and cannot deactivate genes (Lengauer *et al.*, 1997a). It is not yet known which pattern is 'normal', nor whether the phenotype is a direct result of mismatch repair deficiency, but it is clear that inability to undergo appropriate DNA methylation could have enormous consequences for control of cell growth.

#### ***1.3.11.4. Mouse models of mismatch repair gene defects***

Mice lacking *Msh2* are healthy at birth and fertile, but in the first year develop RER+ tumours, mainly metastasizing lymphomas of T-cell origin (de Wind *et al.*, 1995; Reitmar *et al.*, 1995). Later in adulthood, most mice develop intestinal tumours and some also develop skin tumours (Reitmair *et al.*, 1996a). In culture, cells from these mice show microsatellite instability and lose heterology-dependent suppression of recombination. Thus, the murine model supports the hypothesis that *Msh2* mutation contributes to genomic instability in two ways, firstly, by elevation of the rate of point mutations by failure to bind and correct mismatched nucleotides, and secondly, by promoting chromosomal rearrangements through

loss of recombination specificity. A further feature of *Msh2*<sup>-/-</sup> cells is their 20-fold resistance to the methylation agent MNNG compared with wild type cells, discussed below.

Mice deficient in *Msh6* develop a spectrum of tumours similar to that of *Msh2*-deficient mice, but at a later stage in development, with 65% dead by 12 months (Edelmann *et al.*, 1997). Cells from these mice are deficient in single nucleotide mismatch repair but not in repeat insertion/deletion repair, and therefore tumours from these mice do not develop microsatellite instability.

Like *Msh2* null mice, *Pms2*-deficient mice are prone to sarcomas and lymphomas, with 40% developing tumours within the first year (Baker *et al.*, 1995). Tumour tissue exhibits MSI and evidence from the examination of two animals suggests that up to 8.9% of normal cells (including germ cells) are also genetically unstable. The lack of gut tumours in these animals may simply be because sarcomas and lymphomas develop earlier and, given time, *Pms2*-deficient mice would follow a similar course to *Msh2*-deficient animals. However, at least one mechanism of tumorigenic development which occurs in humans, removal of TGF $\beta$ -mediated growth restraint through mutation of a repetitive sequence within the TGF $\beta$ -receptor subunit II gene (Markowitz *et al.*, 1995), is not effective in mice because the murine TGF $\beta$ RII gene does not contain a repetitive sequence. Whilst *Pms2*-deficient females are fully fertile, males are infertile, exhibiting a low sperm count and producing only grossly abnormal spermatozoa. This is thought to be due to aberrant homologue pairing at meiosis I, a phenotype consistent with the analogous phenotype in *S.cerevisiae*.

Mice deficient in *Mlh1* exhibit microsatellite instability in normal tissue (Baker *et al.*, 1996; Edelmann *et al.*, 1996) and are probably susceptible to tumours (though in these reports the mice were too young to be sure). Both sexes are infertile; spermatocytes exhibit high levels of prematurely separated chromosomes, arresting in the first division of meiosis, whilst oocytes appear normal, but fail to develop beyond the single cell stage. Since *Mlh1* was found to localise to sites of crossing over on meiotic chromosomes, the evidence suggests that *Mlh1* exerts its effect on chromosome stability by facilitating the formation or stabilisation of normal levels of chiasmata.

#### ***1.3.11.5. Methylation tolerance in mismatch repair deficient cells***

Mismatch repair-deficient cell lines derived from human and murine RER<sup>+</sup> tumours demonstrate marked resistance to methylating agents such as N-methyl-N'-nitro-N-nitrosoguanine (MNNG) (Parsons *et al.*, 1993; Bhattacharyya *et al.*, 1994; Aquilina *et al.*, 1995; Branch *et al.*, 1995; Reitmaier *et al.*, 1995; de Wind *et al.*, 1995). The sensitivity of normal cells to methylating agents is attributed to successive rounds of unsuccessful mismatch repair. Unless methylated guanine residues (O<sup>6</sup>-MeG) are repaired, the mismatch repair system attempts to repair G-T mispairs but replaces mispaired thymines with the same and a

single-strand nick occurs where repair cannot be completed. Replication results in double-strand breaks and subsequent cell death. O<sup>6</sup>-MeG residues are normally repaired by the methyl-guanine methyl transferase (MGMT) enzyme, and it is interesting to note that the colon has low levels of MGMT, perhaps one reason for the tissue-specificity of HNPCC.

#### **1.3.11.6. Replication errors in the normal tissue of HNPCC patients**

Most HNPCC patients do not exhibit replication errors in their normal tissue, even under conditions that increase requirement for repair (Parsons *et al.*, 1995; Tomlinson *et al.*, 1997). However, in one study, single-cell amplification of microsatellite sequences identified two patients, one with a hPMS2 mutation and one with a hMLH1 mutation, in which a significant proportion of non-neoplastic cells exhibited replication errors (Parsons *et al.*, 1995). Furthermore, replication error-induced mutation appeared to be partially tissue-specific; colonic and urinary tract epithelia from the hPMS2-deficient patient demonstrated a much higher error rate than did non-epithelial colonic tissue. Analysis of normal tissue from other patients with known constitutive mismatch repair gene mutations, including one patient with a different hMLH1 mutation and others with mutations in hPMS1 and hMSH2, revealed no such instability. These results have clear clinical implications; therapeutic agents which target mismatch repair-deficient cells would probably be lethal to non-tumorigenic cells in a subset of patients.

It is puzzling that patients with such compromised mismatch repair, as well as mice in which mismatch repair is totally lacking, do not develop large numbers of tumours as mutations accumulate, as would be expected if accumulation of mutations were the only rate-limiting step. Exposure to mutagens at doses sufficient to induce a comparably high mutation rate would be expected to result in large numbers of tumours. Kinzler and Vogelstein (1996) propose that this may be because mutations occurring in growth-controlling genes as a result of a mutator phenotype produce conflicting signals within the cell, resulting in death of the cell by apoptosis. They suggest that, in contrast, exposure of the gut to mutagens causes not only mutation but also substantial cell death, and any regeneration which follows gives rise to cells in which the apoptosis-promoting signals are necessarily switched off. It is these cells which are susceptible to uncontrolled growth if they acquire, or have previously acquired, mutations in growth-controlling genes. Thus, the authors speculate that irritant, rather than mutagenic, dietary substances may be largely responsible for colorectal carcinogenesis, through induction of tissue regeneration. Changes in dietary habits, especially decreased intake of fibre, which may absorb irritants, could account for the gradual lowering in age of onset of HNPCC.

#### **1.3.11.7. The genetic basis of microsatellite instability in sporadic colorectal cancer**

MSI is generally found to occur in around 15% of sporadic colorectal cancers (Lothe *et al.*, 1993; Young *et al.*, 1993b; Kim *et al.*, 1994a; Aaltonen *et al.*, 1994; Bubb *et al.*,



1996 and Chapter 3), though has been reported in as many as 28% of sporadic colorectal cancers (Thibodeau *et al.*, 1993). It is rarely observed in sporadic colorectal adenomas, and has thus been associated with the transition from adenoma to carcinoma in this disease (Aaltonen *et al.*, 1994; Young *et al.*, 1993). Very striking parallels exist between clinical, pathological and genetic features of such tumours and those from HNPCC patients, suggesting that the diseases may share a common genetic basis. These features are discussed in detail in chapter 3, but, most importantly, include a tendency to be located in the proximal colon, show characteristic histological features, have diploid or near-diploid karyotype, show a negative correlation with abnormalities of p53 and demonstrate a marked increase in patient survival. Furthermore, the incidence of MSI is high (58%) in patients 35 years of age and under with apparently sporadic cancer, indicating a possible genetic basis to a proportion of the cancers occurring within this group (Liu *et al.*, 1995a). Mutation analysis has so far demonstrated few mutations of the mismatch repair genes frequently involved in HNPCC development in RER+ sporadic cancers (Liu *et al.*, 1995b; Borresen *et al.*, 1995; Bubb *et al.*, 1996; Konishi *et al.*, 1996; Moslein *et al.*, 1996; Herfarth *et al.*, 1997). Although hMLH1 and hMSH6 do account for a proportion of sporadic cases (Herfarth *et al.*, 1997; Drummond *et al.*, 1995; Palombo *et al.*, 1995; Papadopoulos *et al.*, 1995), the dearth of mutations of the genes principally responsible for HNPCC indicates either inappropriate mutation detection techniques or the involvement of further components of mismatch repair in RER+ sporadic cancer.

#### ***1.3.11.8. Defective mismatch repair as a mechanism of colorectal tumorigenesis***

The high levels of deletion/insertion mutation associated with defective mismatch repair may facilitate tumour progression by mutating other oncogenes or tumour suppressor genes. Several genes show evidence of a substantially increased mutation rate in cancers with defective mismatch repair. The TGF $\beta$  receptor subunit II is very often disrupted by mutations in an intragenic repetitive tract (Markowitz *et al.*, 1995; Togo *et al.*, 1996) whilst about half of all such tumours have mutation of a polyguanine tract in the apoptosis-promoting gene Bax (Rampino *et al.*, 1997). Mutations of this sort also occur in a polynucleotide tract of hMSH6 in about 30% of RER+ cancers, although the significance of these mutations to mismatch repair is not known (Malkhosyan *et al.*, 1996a). A relatively high number of mutations at repetitive nucleotide units is associated with mutator phenotype status in the p53 gene (Jego *et al.*, 1993; Greenblatt *et al.*, 1996; Sood *et al.*, 1997), the Hprt locus (Eshleman *et al.*, 1996; Malkhosyan *et al.*, 1996b), the insulin-like growth factor II receptor (IGFIIR) (Souza *et al.*, 1996) and APC (Huang *et al.*, 1996b). Mutations of this type in APC are particularly intriguing since APC is the earliest known genetic lesion to occur in sporadic tumours. This implies that an underlying and undetectable low-level defect of mismatch repair may be



present in some individuals, causing APC mutation early in development, perhaps resulting in mutational mosaicism. This hypothesis is supported by the observation that extracolonic features of FAP (caused by germ-line mutations in the APC gene) are present in a significantly higher proportion of patients with microsatellite-unstable sporadic colon cancers than stable tumours (Dunlop *et al.*, 1996).

### 1.3.12. Chromosomal aberrations in colorectal cancer

#### *1.3.12.1. Cytogenetic studies of sporadic colorectal cancer*

The majority of sporadic colorectal cancers display a grossly abnormal chromosome content. Cytogenetic studies and, recently, comparative genomic hybridisation, have identified the most common clonal changes to be loss of chromosomes 17p and 18, whilst loss of chromosomes 1(p3), 4q, 5(q2), 8p, 9p, 10(q2), 14, 15, 21 and Y, and gain of chromosomes 7, 8q, 13, 20 and X are also widely reported (Reichmann *et al.*, 1981; Muleris *et al.* 1988 & 1990; Yaseen *et al.*, 1990; Konstantinova *et al.*, 1991; Bardi *et al.*, 1993b; Barletta *et al.*, 1993; Herbergs *et al.*, 1994; Gerdes *et al.*, 1995; Schlegel *et al.*, 1995; Ried *et al.*, 1996). Structural abnormalities are common, most often involving chromosomes 1, 5 and 17 (Gerdes *et al.*, 1995), although no specific structural abnormality is prevalent in colon cancer and balanced rearrangements are rare (Muleris *et al.*, 1990).

There is a striking progressive increase in chromosomal number and structural rearrangement in the transition from proximal to distal large bowel (Reichmann *et al.*, 1982; Muleris *et al.* 1990; Bardi *et al.*, 1995). Classification of tumours according to the chromosomal abnormalities they possess suggests that at least two distinct types of abnormal karyotype arise, possibly indicating the presence of separate underlying mechanisms of tumour development (Reichmann *et al.*, 1982; Muleris *et al.*, 1988). These groups have been termed 'Monosomic type' (MT) and 'Trisomic type' (TT) by Muleris *et al.* (1990). MT tumours comprise 70% of colorectal cancers, and are characterised by loss of chromosome 18 and 17p, ability to undergo endoreduplication, low expression of the thymidylate synthase (TS) gene (Lasserre *et al.*, 1994), stable microsatellite sequences (Remvikos *et al.*, 1995), frequent abnormal p53 function (Remvikos *et al.*, 1997) and location in the distal colon or rectum. In contrast, TT tumours characteristically gain whole chromosomes but never undergo loss of both chromosome 17p and 18 or endoreduplication, occur predominantly in the rectum, frequently display microsatellite instability and have high TS expression (Lasserre *et al.*, 1994). Additionally, a small proportion (around 7%) of cancers have apparently normal karyotype (termed NT), and are associated with significantly lower age of diagnosis than those with abnormal karyotype, proximal location in the bowel, microsatellite instability (Remvikos *et al.*, 1995; Schlegel *et al.*, 1995) and improved patient survival (Wolley *et al.*, 1982; Kouri *et al.*, 1990; Armitage *et al.*, 1990; Rognum *et al.*, 1991). The mechanisms

driving each type of karyotypic evolution are not understood, but may reflect differences in local environment such as carcinogen content throughout the colon, or, since endoreduplication can occur as a consequence of hypoxic conditions, degree of angiogenesis (Löffler *et al.*, 1987). Alternatively, differences in fundamental molecular characteristics associated with each karyotypic group may drive the acquisition of different chromosomal abnormalities.

#### *Chromosomal abnormalities in adenomas*

Flow cytometry detects aneuploidy in 6% of adenomas (Quirke *et al.*, 1986), whereas more sensitive cytogenetic and *in situ* hybridisation techniques detect clonal abnormalities in half to two-thirds of all adenomas (Reichmann *et al.*, 1985; Longy *et al.*, 1990 & 1993; Griffin *et al.*, 1993; Muleris *et al.*, 1994a; Ried *et al.*, 1996). Karyotypic abnormalities are more pronounced in larger adenomas (Quirke *et al.*, 1986; Longy *et al.*, 1993; Ried *et al.*, 1996), and these abnormalities tend to follow a TT pattern (Muleris *et al.*, 1994a), suggesting that TT carcinomas develop from adenomas more frequently than do MT cancers. Histological evidence refutes this hypothesis, as the proportion of cancers containing remnants of adenomatous tissue reflects the distribution of NT, MT and TT cancers (Muleris *et al.*, 1994a). Rather, MT cancers which arise from adenomas may develop new chromosomal aberrations, whilst TT cancers maintain their original adenomatous pattern of karyotypic abnormality.

Trisomy 7 is a frequent abnormality associated with both malignant and adenomatous proliferating cells, but has also been shown to occur in several cell types *in vitro*, including cells from the stroma adjacent to colonic tumours (Bardi *et al.*, 1993b). This may be a feature of cells from the immune system participating in host response to the tumour (Bardi *et al.*, 1993b), an hypothesis supported by the fact that it is detectable in subpopulations of tumour-infiltrating lymphocytes in renal tumours and surrounding kidney tissue (Dal Cin *et al.*, 1992).

#### **1.3.12.2. Mechanisms of chromosomal instability**

Defects in DNA replication, DNA repair, telomere stability or chromosome segregation may all potentially result in chromosome instability. Once the process has been initiated, resultant chromosomal breakage or aberrant gene expression through mutation, amplification, gene fusion or changes in regulatory control may result in self-perpetuation of the instability state. Possible mechanisms by which instability occurs are described briefly below.

#### *Numerical chromosome changes*

Degeneration of p53-mediated cell cycle control is thought to be an important means by which cancer cells can achieve aneuploidy, structural abnormalities and amplifications.

The process by which loss of p53 function leads to numerical chromosomal instability, probably involving a spindle checkpoint through associations with microtubules and centrosome control, has already been discussed (1.3.2.1 & 1.3.2.2). However, a further mechanism of numerical chromosome aberration which is suggested to be p53-independent has recently been described (Lengauer *et al.*, 1997b). Lengauer *et al.* discovered that, *in vitro*, cells from most colorectal tumours generate chromosome gains or losses as a dominant trait at a high rate, estimated to be  $10^{-2}$  per chromosome per generation (equivalent to a gain or loss every 5 cell generations). The mechanism by which this occurs is not yet known, and although it does not appear to correlate absolutely with p53 status, the presence of a novel mechanism of numerical chromosome instability remains to be proved categorically.

#### *Structural chromosome changes*

McClintock (1951) described two closely-related methods by which structural chromosomal instability can occur. In the first, the chromatid type breakage-fusion-bridge (BFB) cycle, newly broken chromatid ends can undergo sister chromatid fusion, giving rise to a chromatid bridge configuration at anaphase. Further cycles of breakage, fusion and bridge formation ensue until the broken end 'heals', presumably by the chance arrival at the broken end of a stabilising sequence such as telomere-like repeats. In a related process, the chromosome type breakage-fusion-bridge cycle, formation of a dicentric chromosome through aberrant recombination at or near telomeres results in similar formation of an anaphase bridge, repeated breakage and recombination until the chromosome becomes stable.

Several events may initiate the BFB cycle. Firstly, it may occur through the acquisition of DNA strand breaks, in which case abrogation of normal p53 function, with resulting loss of appropriate G1 cell cycle arrest, apoptotic response, and fully functional DNA repair and replication, may play an important role. Defective components of nucleotide excision repair, such as the genetic defect present in patients with Bloom's syndrome, can also result in high levels of DNA breaks and genomic instability (Korn & Ramkissoo, 1995). Secondly, there is evidence that intra-chromosomal telomere-like sequences are hotspots for recombination, breakage and chromosome fusion events that may initiate instability (discussed in Hastie & Allshire, 1989 and Morgan *et al.*, 1996). Thirdly, loss of telomeric sequences might initiate structural chromosome instability. Telomeres comprise multiple stretches of G-rich repeats that function in chromosome replication and protect against end-to end fusions. In germ cells, telomere length is maintained by the enzyme telomerase, but in all other somatic tissue the gene encoding this enzyme is not expressed (Kim *et al.*, 1994b), resulting in progressive reduction in telomere length with age (Hastie *et al.*, 1990). It is thought that severely shortened telomeres may signal a cell to enter senescence, providing a 'mitotic clock' which counts the number of cell divisions. Telomere length is substantially reduced by about

the same extent in both colorectal adenomas and colorectal carcinomas when compared to normal colonic and blood tissue (Hastie *et al.*, 1990), potentially increasing the risk of chromosome fusions, dicentric formation, and subsequent acquisition of the breakage-fusion-bridge cycle. However, maintenance of telomere length to some degree could be a critical event in the progression to colorectal malignancy, allowing cells to avoid the acquisition of a senescence signal associated with substantially shortened telomeres. Expression of telomerase, though not present in colorectal adenoma, is detectable in colorectal carcinoma (Chadeneau *et al.*, 1995) and recent evidence suggests that telomere length can be maintained in tumour cells without apparent telomerase activity (Bryan *et al.*, 1997).

### *Gene amplification*

In mammalian cells, the ability to amplify genes is restricted to genetically unstable transformed or tumorigenic cells (Otto *et al.*, 1989; Tlsty *et al.*, 1990) and is a recessive trait (Tlsty *et al.*, 1992). Cytologically, amplified sequences may be present either intrachromosomally, as homogenously-staining regions (HSR's) or extrachromosomally, in the form of double minutes (DM's). Both DM's and HSR's are rare in primary bowel cancers (Ms M.Gerbault-Seureau, personal communication), although presence of DM's has been demonstrated *in vitro* in one study (Brüderlein *et al.*, 1990) and metastases of colorectal cancers often contain DM's (Reichmann *et al.*, 1981).

In mammalian cells, sequences that contain amplified genes are not simple repeats and often appear at a new chromosomal locations, suggesting that their generation involves several steps (Stark & Wahl, 1984). Several models for gene amplification have been proposed. The unequal exchange model proposes that recombination between DNA sequences on two misaligned chromatids results in a duplication within one chromatid and a deletion within other. Subsequent events are required to resolve the structure, which then becomes stable (Stark & Wahl, 1984). However, this model does not predict that movement of DNA should be associated with amplification. The rereplication model proposes that multiple initiation of DNA synthesis during one cell cycle lead to an 'onionskin' structure which contains many copies of the same DNA sequences (Stark & Wahl, 1984). Such multiple initiations of replication have not been observed in mammalian cells (Windle & Wahl, 1992). A further model suggests that DNA breakage can induce the breakage-fusion-bridge cycle, leading to chromosomal instability and gene amplification. Relaxation of cell cycle checkpoints, such as in p53-deficient cells, does indeed result in generalised genomic instability including an ability to undergo gene amplification (Livingstone *et al.*, 1992; Yin *et al.*, 1992). The breakage-fusion-bridge series of events appears to predominate in rodent cells selected for drug-induced amplification of the dihydrofolate reductase gene (DHFR), adenylate deaminase (AMPD) and CAD, suggested by the observation that telomeric and centromeric sequences are found close

to amplified regions within rearranged chromosomes (Bertoni *et al.*, 1994). Alternatively, the events following DNA breakage may include the generation of small acentric molecules that can increase in copy number through unequal segregation on cell division (Windle *et al.*, 1991), and there is evidence that extrachromosomal amplification can precede integration into the genome (Stark *et al.*, 1989 & 1993; Wahl *et al.*, 1989; Hamlin *et al.*, 1991). Hypoxia, which occurs when tumours outgrow their vascular supply, has been demonstrated to facilitate gene amplification (Rice *et al.*, 1986), and this has been postulated to be due to the induction of endonuclease activity, which may potentiate break-related genomic instability (Stoler *et al.*, 1992; Russo *et al.*, 1995).

## 1.4 Summary

Colorectal cancer arises as a result of a complex series of interactions between dietary and genetic factors. This chapter has discussed current knowledge of aetiological factors, the genetic abnormalities characteristic of colorectal cancer and the mechanisms involved in initiating and perpetuating instability within the cancer genome. The genes known to be involved in colorectal cancer development are summarised in Table 1, overleaf.

Several lines of evidence suggest that sporadic colorectal cancers evolve along different pathways according to the type of underlying genomic instability they possess. On the basis of clinical, pathological and molecular features, two general groups can be defined, one characterised by widespread microsatellite instability, low levels of p53 abnormality, near-diploid DNA content, proximal location, mucinous histology and better prognosis, the other tending toward abnormal chromosome content, abnormalities of p53 function, distal location and poor prognosis. Tumorigenesis in the first cancer type is thought to occur because of defects of mismatch repair, whilst there is evidence that the second may be driven by abnormalities of p53 (Carder *et al.*, 1993 & 1995). Investigation of the nature and coexistence of these types of instability forms the basis of this thesis.



**Table 1**

**Summary of gene defects in sporadic colorectal cancer**

<b>Genetic event</b>	<b>Proportion of sporadic colorectal cancers affected</b>	<b>Possible role in cancer cell</b>
<b>Methylation changes</b>	100%	Up-regulation of oncogenes, down-regulation of tumour suppressor genes
<b>APC mutation or loss</b>	>60% (but possibly nearer 100%)	Disruption of cell adhesion and possibly intercellular communication and cell migration
<b>p53 mutation, deletion or abnormally stabilised protein</b>	75%	Loss of control of genomic stability through loss of cell cycle control, apoptosis, DNA replication and repair
<b>Loss of DCC transcript</b>	56% (but 70% deletion of 18q where DCC located)	Disruption of cell adhesion?
<b>DPC4 mutation JV18 mutation MADR2 mutation</b>	28% 11% 6%	Loss of TGF $\beta$ -mediated growth control
<b>Ki-ras mutation</b>	50%	Disruption of signal transduction
<b>c-myc overexpression</b>	50%	Increased cell proliferation
<b>Defects of mismatch repair</b>	15%	Loss of control of genomic stability through loss of replication fidelity
<b>TGF<math>\beta</math>RII mutation</b>	60% of RER+ cancers	Loss of TGF $\beta$ -mediated growth control
<b>Bax mutation</b>	>80% of RER+ cancers	Loss of apoptosis promotion

### 1.5. Aims of this study

The aim of this project was to investigate aspects of the microsatellite instability phenotype, its genetic basis, and its interaction with chromosomal instability and defects of p53. Firstly, the microsatellite instability phenotype was studied in more detail. Its prevalence was determined in a large series of sporadic colorectal cancers from a Scottish population, and related to clinical and pathological features in order to try to define a group of cancers with a distinct biological basis. Also, the gene defect underlying this phenotype was investigated by mutation analysis of a likely candidate gene, hMSH2. Secondly, the behaviour of stable and unstable microsatellites over time was then studied *in vivo* through analysis of tumours grown as xenografts in mice. These investigations are recorded in chapters 3 and 4. Thirdly, the coexistence of microsatellite instability and p53 defects with chromosomal abnormalities in human sporadic colorectal cancer was examined. Analysis of patterns of chromosomal change was undertaken using Comparative Genomic Hybridisation (CGH), a powerful DNA analysis technique which detects regions of genomic amplification or deletion by simultaneous hybridisation of differently-labelled DNA derived from normal and test tissue onto a normal metaphase spread (Kallioniemi *et al.*, 1992; du Manoir *et al.*, 1993). A fluorescence ratio along the length of the chromosome is generated and changes in chromosome copy number are detected as a change in the ratio of green to red fluorescence. This technique was used in order to try to define, firstly, the number of chromosome abnormalities associated with each of these putative pathways of tumorigenesis and, secondly, any unique patterns of chromosomal gain or loss associated with each molecular defect. Tumours employed in this study were grown for one or two passages as subcutaneous xenografts in SCID mice in order to eliminate stromal contamination. Similar methods were then applied to a related study of transgenic mice lacking p53 and/or the mismatch repair gene *Msh2*, in order to define levels of chromosomal change and MSI associated with these defects in a system lacking the complicating factors present in human tumours.

A deeper understanding of these mechanisms of instability and their interactions is crucial to ascertaining the nature and extent of heterogeneity within this disease. In the short term, this may prove to be clinically important as it could help to determine appropriate chemotherapeutic treatment, avoiding ineffective or harmful approaches. In the longer term, it may provide novel targets on which base both new drugs and gene therapy.

## CHAPTER 2

### MATERIALS AND METHODS

Suppliers of reagents are listed in appendix I and II.

#### 2.1 Tissue samples

Tissues were harvested fresh from consecutive sporadic colorectal carcinomas removed at operation between 1988-1994 at Edinburgh Royal Infirmary, and frozen at  $-70^{\circ}\text{C}$ . For all samples, representative portions of tumour were paraffin-processed and stained with haematoxylin and eosin in the Department of Pathology, University of Edinburgh, following standard methodology. DNA was extracted as in 2.3.1, and xenografts were established whenever resources permitted according to the method described below. Relevant clinical, pathological and genetic details for primary and xenografted tumours included in this study are given in Appendix 5.

#### 2.2 Establishment of xenografts

Xenografted tumours were grown subcutaneously in severe combined immunodeficiency syndrome (SCID) mice. These mice are deficient in both B and T lymphocyte function due to an inability to correctly rearrange the immunoglobulin and T-cell receptor genes by site-specific [V(D)J] recombination (Bosma *et al.*, 1983), and thus do not reject implanted foreign material. SCID mice do, however, retain non-specific macrophage-mediated immune response, useful for removal of bacterial contamination from implanted tumours.

Xenografts were established by implantation of two pieces of primary tumour  $\leq 5\text{mm}^3$ , first rinsed in cold wash solution (Glasgow medium supplemented with penicillin, streptomycin, HEPES and 10% serum), through a small dorsal incision. Tumours were allowed to grow until an externally visible diameter of about 1cm was reached, or when signs of poor health were apparent, a usual duration of 28-56 days. Mice were then killed and tumours either snap frozen in liquid nitrogen, frozen for future passage in a Glasgow medium/dimethyl sulphoxide (DMSO) mix, or immediately implanted into another mouse.

#### 2.3 Extraction of DNA

##### 2.3.1 DNA extraction from frozen tissue

DNA was extracted from frozen tissue according to the method of Goelz *et al.*, (1985b). Frozen tissue was chopped into small pieces in 0.5ml TE-9 SDS and 0.5mg/ml proteinase K and incubated for 48 hours at  $48^{\circ}\text{C}$ , mixing occasionally. An equal volume of water-saturated phenol was added, mixed and centrifuged at  $10000 \times g$  for 2 minutes. The

top layer was removed and an equal volume of PC-9 added to it, mixed and spun again. The top layer was removed and an equal volume of 24:1 chloroform: isoamyl alcohol was added, mixed and spun. The top layer was once again removed and 0.5 volume 7.5M ammonium acetate and 2x volume ethanol added, mixed and left overnight at -20°C. This was centrifuged at 4°C and 10000 x g for 20 minutes, the supernatant drained and the pellet vacuum dried. The DNA was resuspended in 250µl TE, dissolved at 4°C for 3 hours and an OD reading taken at 260nm. Concentration was adjusted to 1mg/ml.

TE-9 SDS:	PC-9:	TE:
500mM Tris pH8	50ml water-saturated phenol	10mM Tris.
20mM EDTA	50ml chloroform	1mM EDTA.
10mM NaCl		
1% SDS		

### 2.3.2 DNA extraction from blood

Blood samples were collected in 1% EDTA to prevent clotting. Blood was lysed in an equal volume of lysis buffer for 30 minutes at room temperature, and equal volume of water-saturated phenol added, mixed and centrifuged at 10000 x g for 20 minutes. The top layer was removed and 0.5 x volume 7.5M ammonium acetate and 2 x volume cold isopropanol added. This was left at -20°C overnight, spun at 8000 x g for 30 minutes and the pellet drained until dry. The pellet was then resuspended in 250µl TE, dissolved for 3 hours at 4°C and an OD reading taken at 260nm. Concentration was adjusted to 1mg/ml.

Lysis buffer:	TE: see 2.3.1
0.1M Tris pH8	
20mM NaCl	
1mM EDTA	
4% SDS	

## **2.4 Agarose gel electrophoresis**

Agarose gels were prepared by adding 3g electrophoresis-grade agarose to 150ml 1x Tris-borate-EDTA buffer (TBE) and heating to boiling in a microwave oven, with regular mixing, to dissolve the agarose. The gel was stirred and allowed to cool to approximately 55°C before adding 20 ng/ml ethidium bromide and pouring into a 12x16cm gel mould with a 20-well comb. For checking PCR products, 8µl product was added to 1µl loading dye before loading. Products were run against 500ng of a suitable molecular weight marker, usually

marker V (Boehringer-Mannheim Ltd). For larger fragments,  $\phi$ X174 RF DNA/*Hae*III or 1kb ladder (Life Technologies Ltd) were used. Where quantitation of the product was required, products were run against 4 $\mu$ l DNA mass ladder (Life Technologies Ltd). Details of molecular weight markers are given in Appendix 3. Electrophoresis was carried out at 125V in 1xTBE buffer for variable lengths of time; for most purposes, migration of the bromophenol blue dye approximately 5cm through the gel was sufficient. Gels were visualised and photographed on a UV box.

10xTBE:	Loading dye:
107.8g Tris (0.89M)	30% glycerol
55g Boric acid (0.89M)	0.25% bromophenol blue
7.44g Na <sub>2</sub> EDTA (0.02M)	0.25% xylene cyanol FF
in 1l DDW	
pH adjusted to 8.3	

## 2.5 Flow cytometry

### 2.5.1 Preparation of cells

Tissue was prepared for flow cytometry according to the method of Vindelov *et al.* (1983). Tumour samples approximately 4mm<sup>3</sup> were macerated using a sterile scalpel and resuspended in 200 $\mu$ l citrate buffer. 100 $\mu$ l of this solution was added to 450 $\mu$ l Solution A to lyse the cells, mixed gently and incubated at room temperature for 10 minutes. This solution was then neutralised by incubation for 10 minutes at room temperature with 325 $\mu$ l of solution B, and cells were stained by the addition of 250 $\mu$ l of propidium iodide, Solution C, on ice for 10 minutes. The cell solution was then drawn through a syringe needle several times to disperse large clumps of cells. Samples were stored on ice and analysed within 8 hours.

Solution A:	Solution B	Solution C:
15mg trypsin	250mg trypsin inhibitor	208mg propidium iodide
500ml stock solution	50mg ribonuclease A	500mg spermine
pH7.6	500ml stock solution	tetrahydrochloride
	pH7.6	500ml stock solution
		pH7.6

Citrate buffer:

85.5g sucrose

11.76g trisodium citrate

dissolved in 800ml DDW

50ml DMSO added and volume made to 1litre with

DDW, pH 7.6.

Stock solution:

2g trisodium citrate

121mg Tris

1.044g spermine

tetrahydrochloride

2ml Nonidet P40

in 2l DDW, pH 7.6

### 2.5.2 Flow cytometry and analysis

Flow cytometry was performed on a Coulter EPICS-XL flow cytometer at an excitation wavelength of 488nm. Before each use, the alignment of the laser was checked using Immuno-Check alignment fluorescent spheres (Coulter Electronics Ltd). Half-peak coefficients of variation were typically less than 2%. At least 10,000 nuclei were analysed in each sample, and tumour DNA content estimated by comparison with identical analyses of normal tissue.

### **2.6 Immunocytochemical detection of stabilised p53**

Stabilised p53 protein can readily be detected by immunohistochemical methods, whereas the short half-life of the wild-type protein normally renders it undetectable by such methods (Oren *et al.*, 1985). Immunocytochemistry was carried out using two antibodies, pAb1801 and DO7. The pAb1801 epitope is located between amino acids 45 and 91, and the antibody is capable of reacting with both wild-type and mutant p53. DO7 also recognises an amino terminal epitope and reacts similarly.

Slides were prepared in the Department of Pathology, University of Edinburgh, by cutting 3µm sections from tissue stored in periodate-lysine-paraformaldehyde-dichromate (PLPD) and embedded in paraffin wax. Sections were placed onto poly L-lysine (PLL)-treated microscope slides.

Samples were deparaffinised by placing in xylene for 10 minutes, absolute ethanol for 1 minute, 74OP for 1 minute and 64OP for 1 minute and then rinsed in water. Endogenous peroxidase activity was blocked by treatment in 3% hydrogen peroxide for 15 minutes. This was followed by washing for 5 minutes in DDW and 5 minutes in phosphate buffered saline (PBS). Slides were then wiped to remove excess fluid and marked around the area of the section with a wax pen to retain solutions. 100µl normal rabbit serum (NRS) diluted 1/5 in PBS was added and left for 20 minutes. This was then drained and the slides incubated



overnight at 4°C in a 1/100 dilution of either Do-7 or pAb1801 antibody in 1/5 NRS/PBS solution.

After overnight incubation, the slides were washed twice for 5 minutes in PBS before applying the secondary antibody. Biotinylated rabbit anti-mouse immunoglobulins (Dako Ltd) were diluted 1/400 in the above NRS/PBS solution and 100µl added to the slide and incubated for 30 minutes at room temperature. Meanwhile, an avidin/biotinylated horseradish peroxidase (HRP) complex (ABC kit, Dako Ltd) was made up by adding 1 drop of avidin and 1 drop of biotinylated horseradish peroxidase to 5ml PBS and allowing 30 minutes for the reagents to conjugate. After incubation, the slides were washed twice in PBS for 5 minutes and 100µl ABC complex was added. Slides were incubated for 30 minutes at room temperature then again given two 5 minute washes in PBS. They were then incubated for 3 minutes in diaminobenzidine (DAB) solution at room temperature to allow formation of brown colouration through oxidation of DAB by HRP, washed for 5 minutes in DDW and counterstained for 6 seconds in haematoxylin. They were washed until the water was clear and dehydrated through 1 minute in 64OP, 1 minute in 74OP, 1 minutes in absolute ethanol and 10 minutes in xylene. Slides were mounted with DPX mountant and examined under 100x magnification on a light microscope. Slides were scored positive if any nuclei stained positive and extent of staining, which varied considerably, was noted. Although strength of staining also varied, this was not taken into account as it could vary between experiments.

#### PLPD:

0.1M lysine

0.1M periodate

2% paraformaldehyde

made to 50ml with Sorensen's phosphate buffer (0.05M, pH7.4).

5% potassium dichromate in 50ml DDW added immediately before use

#### PBS:

8g NaCl

0.2g KCl

1.44g Na<sub>2</sub>HPO<sub>4</sub>

0.24g KH<sub>2</sub>PO<sub>4</sub>

to 1 litre DDW

pH7.4

#### DAB solution:

5mg DAB

4.8ml DAB buffer

100µl 1/30 hydrogen peroxide solution

#### DAB buffer:

24ml 0.2M Tris

38ml 0.1N HCl

0.0681g imidazole

DDW to 100ml, pH adjusted to 7.6

## 2.7 Analysis of microsatellite instability

### 2.7.1 Polymerase Chain Reaction (PCR) of microsatellite sequences for non-isotopic analysis.

The following microsatellite markers were analysed for evidence of genetic instability in DNA from primary and xenografted tumours: D2S119, D3S1293, D8S282, D13S160, D15S132, D17S849 (Gyapay *et al.* 1994) and are detailed in Table 2. At least four sites were examined in each patient. Reactions were carried out in a 96-well plate and conditions consisted of a final concentration of 1x buffer, 200µM each dNTP, 0.5µM each primer, 0.625 units *Taq* DNA polymerase and 25ng genomic DNA template. Magnesium ion concentrations and annealing temperatures for each primer set are indicated in Table 2. Reaction volume was made up to 25µl with distilled deionised water (DDW) and one drop of paraffin oil was overlaid to prevent evaporation. 30 cycles of amplification were carried out with each cycle consisting of 1 minute denaturation at 95°C, 1 minute annealing at variable temperature (Table 2) and 2 minutes extension at 72°C. Reactions were checked on a 2% agarose gel as in 2.4.

PCR buffer (Buffer IV, Advanced Biotechnologies Ltd):

750mM Tris-HCl (pH9 at 25°C)

200mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>

0.1% w/v Tween

**Table 2**  
**Primer sequences for amplification of microsatellites.**

MARKER NAME	LOCUS	[Mg <sup>2+</sup> ] CONC.	ANNEALING TEMP.°C	APPROXIMATE ALLELE SIZES (bp)	PRIMER SEQUENCES (5'-3')
AFM077yb7	D2S119	2mM	55	214-232	CTTGGGGAACAGAGGTCATT GAGAATCCCTCAATTTCTTTGGA
AFM093xb3	D2S123	2mM	58	210-240	AAACAGGATGCCTGCCTTTA GGACTTTCACCTATGGGAC
AFM200za1	D3S1293	2mM	50	116-144	ACTCACAGAGCCTTCACA CATGGAAATAGAACAGGGT
AFM234vf4	D8S282	1.5mM	50	260-274	GGGCACAGGCATGTGT GGCTGCATTCTGAAAGGTTA
AFM157xa11	D13S160	1.5mM	55	229-241	CGGGTGATCTAAGGCTTCTA GGCAGAGATATGAGGCAAAA
AFM265xf9	D15S132	1mM	55	69-83	CTGATAATAAAACCAGGAAGACAC TATTGGCCTGAAGTGGTG
AFM234wg3	D17S849	1mM	50	144-174	CAATTCTGTTCTAAGATTATTTGG CTCTGGCTGAGGAGGC

## 2.7.2 Analysis of microsatellites by silver staining

### **2.7.2.1 Preparation of denaturing gel**

35x50cm glass plates for vertical gel electrophoresis were thoroughly cleaned with cream cleaner, rinsed well and wiped with 70% ethanol. In order to carry out silver staining *in situ*, the gel was bound to the large glass plate by first treating with  $\gamma$ -methacryloxypropyltrimethoxysilane ('bind silane'). Five microlitres of bind silane was added to 1ml 95% ethanol, 0.05% acetic acid and spread over one plate. This was left to dry for 5 minutes and then cleaned twice with 95% ethanol before pouring the gel. The other plate was treated by wiping with Gel Slick (AT Biochem), leaving for 5 minutes and cleaning with 70% ethanol. 0.4mm spacer and square-well or shark's tooth combs were used. 200 $\mu$ l TEMED and 200 $\mu$ l 10% ammonium persulphate were added to 60ml acrylamide solution immediately before pouring, the gel poured and left to set for at least one hour. The combs were removed, the wells rinsed with 0.5x TBE and the gel prerun at 70W in 0.5x TBE for one hour prior to loading in order to heat to about 55°C.

Acrylamide solution:

75ml 19:1 acrylamide:bis acrylamide 'Instagel' (Severn  
Biotech Ltd)

250g 500ml urea (7M)

50ml 10x TBE

175ml DDW

stored protected from light at 4°C

1 x TBE buffer:

see 2.4

### **2.7.2.2 Electrophoresis**

Five microlitres of PCR product was denatured at 95°C for 5 minutes in 0.5X volume short tandem repeat (STR) loading solution on a thermal heating block then placed on ice. After rinsing the wells of the polyacrylamide gel with 0.5X TBE, 6 $\mu$ l of treated PCR product were loaded into each well and electrophoresis carried out at constant power (70W) until the xylene cyanol marker in the loading dye reached to bottom of the gel (about 4 hours). Optimal running temperature was 50-55°C.

STR loading solution:

10mM NaOH

95% formamide

0.05% bromophenol blue

0.05% xylene cyanol FF

### 2.7.2.3 Silver staining of microsatellite gels

Gels were stained on a rocking platform according to the method of Bassam *et al.* (1991), as detailed in Promega's DNA Silver Staining System protocol. Ultrapure DDW (UDDW) was used throughout. The plates were separated and the gel, attached to one plate, was placed in fix solution for 20 minutes, washed three times in UDDW for 2 minutes and placed in staining solution for 30 minutes. The gel was then washed for 10 seconds in UDDW and placed in chilled developer solution until bands appeared. The reaction was stopped with 10% glacial acetic acid for 5 minutes, the gel washed for 2 minutes in UDDW and then read and photographed on a light box. For re-use, plates were soaked in 5M sodium hydroxide for one hour and cleaned with detergent and 70% ethanol. Spent silver nitrate was treated with sodium chloride to precipitate the silver before disposal.

Fix Solution:	Staining Solution:	Developer Solution:
10% glacial acetic acid in Ultrapure water	1g silver nitrate 1.5ml 37% formaldehyde made to 1 litre with Ultrapure water	81g sodium carbonate (decahydrate) 1.5ml 37% formaldehyde 2mg sodium thiosulphate made to 1 litre with Ultrapure water

### 2.7.3 Radioactive detection of microsatellite sequences

#### 2.7.3.1 Radioactive isotope labelling

PCR reactions were carried out in a thermal cycler using 96-well plates. The reactions were labelled with  $\gamma$ -[ $^{33}\text{P}$ ]dATP and the products separated by vertical electrophoresis in 6% denaturing polyacrylamide gel.

Oligonucleotide primer sequences were diluted with sterile distilled water before use, giving a final concentration of 10ng/ $\mu\text{l}$ . The CA strand primer in each reaction was 5' end-labelled with  $\gamma$ - $^{33}\text{P}$  dATP, using T4 polynucleotide kinase. Labelling was performed prior to each PCR reaction. 10ng of oligonucleotide (10ng/ $\mu\text{l}$ ) was added to 2 $\mu\text{l}$  of 5x buffer, 6.2 $\mu\text{l}$  of sterile distilled water, 0.3 $\mu\text{l}$  of  $\gamma$ - $^{33}\text{P}$  dATP (equivalent to 3 $\mu\text{Ci}$ ) and placed on ice. 0.53 $\mu\text{l}$  (5U) of T4 polynucleotide kinase was then added giving a total volume of 10 $\mu\text{l}$  and the reaction placed in a water bath at 37°C for one hour. This amount was sufficient for up to 10 PCR reactions.

5X T4 polynucleotide kinase buffer:  
350mM Tris-HCl pH 7.6

50mM MgCl<sub>2</sub>  
500mM KCl  
5mM 2-mercaptoethanol

### 2.7.3.2 Preparation of PCRs

Polymerase chain reactions were carried out as in section 2.7.1, except that prior to amplification an additional 1µl labelled primer was added to each reaction. After amplification, 5µl stop solution was added to 5µl PCR product, the mixture boiled for 3 minutes and cooled quickly on ice in preparation for loading onto a gel.

Stop solution:

95% formamide  
10mM EDTA pH8  
0.1% bromophenol blue  
0.1% xylene cyanol FF

### 2.7.3.3 Polyacrylamide gel electrophoresis of radio-labelled microsatellite sequences

A 6% denaturing polyacrylamide gel was used in vertical sequencing gel apparatus with 0.5xTBE buffer. Gels were prepared in 35cm x 50cm vertical DNA sequencing electrophoresis plates. Plates were thoroughly cleaned with 'Decon' detergent, rinsed with water and finally wiped with 70% alcohol. One plate was siliconised by wiping with 3ml Gel Slick, air drying and removing excess with water. Plates were assembled with 0.4mm spacers and combs. Sixty microlitres of TEMED and 60µl 25% ammonium persulphate (APS) were added to 60ml stock acrylamide immediately prior to pouring and the gel was allowed to polymerise for 1 hour. The gel was pre-run for an hour in 0.5xTBE at 70W to reach 55°C.

5µl of combined PCR product and stop solution denatured as described above were added to the wells after rinsing with TBE to disperse urea. The gel was then run at 70W for 3-6 hours or until the xylene cyanol marker dye approached the end of the electrophoresis plate. It was then fixed for 10 minutes in a solution of 10% methanol and 10% acetic acid, transferred to Whatman No 17 paper and dried on a vacuum gel drier. Autoradiography was carried out for 1-3 days and autoradiographs were assessed visually for microsatellite instability.

Denaturing polyacrylamide gel:  
see 2.7.2.1

10xTBE:  
See 2.4

Stop solution:  
see 2.7.3.2





#### 2.7.4 Microsatellite analysis with automated laser fluorescence (ALF) DNA sequencer

An Automated Laser Fluorimeter (ALF) DNA sequencer was used in the assessment of microsatellite instability in tumour DNA from primary sporadic colorectal cancers. This work was carried out by Mr C. Cunningham and will be described only briefly.

PCR was carried out under standard conditions with the inclusion of one fluorescently-labelled primer. Appropriate internal standards were added and samples were alkali and heat denatured and rapidly cooled prior to electrophoresis on a 7M urea 6% MDE gel (AT Biochem) using an ALF DNA sequencer. Microsatellite instability in tumour DNA was determined by a shift of the allele peak to a new position when compared with corresponding normal DNA.

### **2.8 Mutation detection in hMSH2**

#### 2.8.1 Mutation detection by single-stranded conformational polymorphism analysis

The *hMSH2* gene covers about 73kb of genomic DNA and spans 16 exons (Kolodner *et al.*, 1994). Primers were designed to amplify each exon separately, including splice sites where possible. Mutation screening was carried out using a combination of SSCP and heteroduplex analysis of PCR-generated DNA fragments. Initially, SSCP analysis was carried out on exons 2-15, for which the intron-exon boundaries were known (Dr K. Kinzler, personal communication). Each exon was amplified separately by PCR using primers which included most 5' and 3' splice sites. Some exons, particularly 3, 12 and 15 proved difficult or impossible to resolve by this method, producing either smearing or only a single band on the gel. The inability of these fragments to form discrete secondary structures that can easily be resolved on an acrylamide gel may be related to the high A-T content of the gene, although the problematic fragments did not differ in their A-T content from other resolvable *hMSH2* PCR fragments. Where SSCP analysis did not produce a satisfactory result, heteroduplex analysis was carried out. Exons 1 and 16, when their sequences became available, were examined by heteroduplex analysis only as this proved to be somewhat faster than SSCP. Samples which showed an abnormal banding pattern by either method were sequenced using a modified dideoxy chain termination method.

##### **2.8.1.1 Polymerase Chain Reaction of the *hMSH2* gene.**

All 16 exons of *hMSH2* were amplified using the primers described in Table 3. Sequences of the intron-exon boundaries were kindly provided by Dr K. Kinzler. Primers were designed to amplify each exon separately, including splice sites where possible. Reactions consisted of 50µl volumes containing 100ng genomic DNA and a final concentration of 0.25 to 0.5µM each primer, 200µM each dNTP, 2.5mM magnesium chloride (except exons 15 and

1, which contained 1.5mM), 1x buffer and solutions were overlaid with one drop of paraffin oil. This was heated to 95°C for 5 minutes, cooled to 80°C and 1.25U of thermostable DNA polymerase added to 'hot start' the reaction, a technique found to significantly improve reaction specificity in many cases. PCR was carried out for 30 cycles at variable annealing temperatures (Table 3) with the initial 2 cycles annealing at higher temperature than subsequent cycles (as described in the footnotes to Table 3) in order to promote specific target amplification. This procedure was found to greatly improve specificity of amplification in most cases.

PCR Buffer:

See 2.7.1

**Table 3****Primer sequences for amplification of hMSH2.**

Exon	Annealing temperature <sup>a</sup> (°C)	Product size (bp)	Primer sequences (5' to 3')
1	44	302	GCGCATTCTTCAACCAGG AAAGGAGCCGCGGCCACAA
2	44	236	ACATGTAATATCTCAAATCTG AAATAAGTAAATTAAAAAGGAAG
3	55	376	AAAGTATGTTCAAGAGTTTGT ATCTCCTCTATCACTAGACT
4	44	212	TTATTCTTTTCTCATAGTAG TTGTAATTCACATTATAATCC
5	46 <sup>b</sup>	241	TGGCTATAGGAAATCTTCG AACCATTCAACATTTTAACC
6	55	225	ACTAATGAGCTTGCCATTC AACTGCAGGTTACATAAAAC
7	44	225	AAATATTTTACATTAATTCAAG GTATGIGTTGAAGGAAAAC
8	49	279	GATCTTTTATTTGTTTG AAAAATAACTACTGCTTA
9	50 <sup>c</sup>	191	GGATTTTGTCACTTTGTTT TAGGACAAAAGAATTATTCC
10	48	195	TGGTAGTAGGTATTTATGG CATTTAGGGAATTAATAAAG
11	54	250	ACTGTTATTTTCGATTTGCAG CCAGGTGACATTCAGAAC
12	54	159	TTTATTATTCAGTATTCCTGTG CCACAAAGCCCAAAAACCA
13	48	325	AGAAAGAAGTTTAAACTTGC CTATCTTCAAGGGACTAGG
14	44	281	TACCACATTTTATGTGATG TACTGAATTTAGAGTACTC
15	48 <sup>d</sup>	325	GCTGTCTCTTCATG TAATAATAGAGAAGCTAAG
16	50	270	AGAIATTTTAATTACTAATGGG AATATCTTATTAAGTTGATAGC

<sup>a</sup> Unless otherwise stated, the first two PCR cycles were carried out at annealing temperatures 2°C higher than the following 28, which were at the temperature stated in table 2.

<sup>b</sup> Annealing for 1 cycle at 52°C followed by stepdown by 1°C each cycle, ending with 26 cycles at 44°C.

<sup>c</sup> Annealing for 1 cycle at 54°C followed by stepdown by 1°C each cycle, ending with 28 cycles at 50°C.

<sup>d</sup> Annealing for 1 cycle at 52°C followed by stepdown by 1°C each cycle, ending with 28 cycles at 48°C.

### 2.8.1.2 SSCP electrophoresis of hMSH2 PCR products

Gels were poured in 16x20cm vertical electrophoresis plates with 0.75mm spacers and 20-well combs. To 30ml 0.5xMDE gel solution, 120 $\mu$ l 10% ammonium persulphate and 12 $\mu$ l TEMED were added, the solution mixed and poured immediately. It was allowed to polymerise at 30°C for one hour. Five microlitres of PCR product were denatured for 5 minutes at 50°C in 1 $\mu$ l denaturing solution. Three microlitres of stop solution were added to each tube and the samples loaded quickly onto the gel, after rinsing the wells with 1xTBE. Electrophoresis was carried out at up to 30W cooled to approximately 20°C by a circulating water bath. Running time varied according to fragment size; good fragment separation was achieved when samples were run such that the xylene cyanol marker dye had migrated between 20 and 60cm through the gel. Electrophoresis buffer was 1xTBE.

0.5xMDE gel solution:	Denaturing solution:	10xTBE:
7.5ml MDE gel	0.5M sodium hydroxide	See 2.4
1.5ml glycerol	10mM EDTA	
3ml 10x TBE		
18ml DDW		

### 2.8.1.3 Silver staining of SSCP gels

Silver staining of SSCP gels was carried out in a shallow glass dish on a gently shaking platform, carefully pouring off used solutions. Ultrapure water was used throughout. Staining was done using the BioRad silver stain kit.

The gel was removed from both plates and placed in 40% methanol for 30 minutes, followed by two 15 minute washes in 10% ethanol. The gel was then treated with oxidiser solution for 3 minutes and then repeatedly washed for two minutes with water until absolutely colourless. It was then placed in staining solution for 15 minutes, washed twice for 30 seconds in water and then treated with developer solution until a brown precipitate formed. The developer was replaced with fresh and the gel left until bands appeared. The reaction was stopped with 5% glacial acetic acid for 5 minutes and the gel transferred onto 3MM paper and dried at 80°C for one hour on a gel dryer.

BioRad silver stain kit components (exact composition not given):

Oxidiser solution:	Staining solution:	Developer solution:
potassium dichromate	silver nitrate solution	sodium carbonate and paraformaldehyde
solution in Ultrapure water	in Ultrapure water	solution in Ultrapure water

#### 2.8.1.4 Restriction enzyme digestion of hMSH2 exon 5 and exon 2 PCR products

Twenty-one products generated from exon 5 were abnormal by SSCP and sequencing revealed changes in the length of a polyadenine tract within intron 5 (see Chapter 3, Results). In order to eliminate the possibility of other mutations in the exon, this portion was cleaved away with *Tru* II and the remaining exonic fragment reanalysed by SSCP. Similarly, 8 products generated from exon 2 which were abnormal by SSCP but revealed only a length change in an intronic polynucleotide tract by sequencing, were cleaved with *Pst* I to allow reanalysis of the exonic portion by SSCP. Reactions were carried out by adding 10U enzyme (usually 1µl) to 10µl PCR product and 1 x concentration of appropriate buffer in a total volume of 30µl, mixing and incubating for at least 2 hours at 37°C for *Pst* I or 65°C for *Tru* II. Samples were then ethanol-precipitated by adding 3 x volume of ice-cold ethanol and one tenth volume of 7.5M ammonium acetate and leaving at -70°C for 30 minutes. They were then centrifuged at 10000 x g for 15 minutes, the supernatant removed and the pellet washed in 70% ethanol. Finally, the pellet was centrifuged at 10000 x g for 15 minutes at 4°C, the supernatant removed, the pellet air dried and resuspended in 10µl TE buffer. Half of this quantity was then used for SSCP analysis as in 2.8.1.2.

10x <i>Pst</i> I buffer:	10 x <i>Tru</i> II buffer:	TE:
500mM Tris-HCl (pH8.0)	100mM Tris-HCl (pH8.5)	10mM Tris pH8
100mM MgCl <sub>2</sub>	100mM MgCl <sub>2</sub>	1mM EDTA
500mM NaCl	1M KCl	
	1mg/ml BSA	

#### 2.8.2 Restriction digest assay for hMSH2 exon 13 polymorphism

A previously-reported polymorphism in hMSH2 intron 12 (Fishel *et al.*, 1993; Hall *et al.*, 1994), a T-C change at the -6 position of the intron 12 3' splice site, was found in three patients by sequencing samples with heteroduplex bands (see Chapter 3, Results). In order to confirm its presence in other samples, a restriction digest assay for the polymorphism was designed. *Sfc* I was chosen for the assay as it cuts at CTPuPyAG; thus, the T-C change creates the site, cleaving 40 base pairs from the 281 base pair product. All DNA samples were checked by this method. One unit of *Sfc* I was added to 5µl PCR product, 1µl 10x buffer, 100 µg/ml bovine serum albumin (BSA) and the volume made to 10µl with DDW. Digestion was allowed to proceed for one hour at 37°C and the reaction was stopped by heating to 65°C for 10 minutes. Digestion products were separated by electrophoresis on a 3% agarose gel containing 50 ng/ml ethidium bromide in 1xTBE against 500ng marker V (Boehringer Mannheim Ltd) and gels photographed on a UV box.

10x *Sfc*1 buffer:  
60mM Tris-HCl pH7.5  
60mM MgCl<sub>2</sub>  
50mM NaCl  
1mg/ml bovine serum albumin  
70mM  $\beta$ -mercaptoethanol  
1mM ATP  
20mM dithiothreitol  
10mM spermidine

### 2.8.3 Restriction digest assay for polymorphism in the hMSH2 untranslated region 5' to the exon 1 coding sequence

A frequent abnormality in the exon 1 PCR fragment was detected by the presence of an extra band by heteroduplex analysis and a consistent SSCP banding pattern (See Chapter, Results, and figure 6). Sequencing revealed a C→G substitution at the +9 position of intron 1, a change which destroys a *Bcn*1 cleavage site. DNA samples from 106 healthy blood donors were screened by restriction enzyme digest assay to ensure that the sequence alteration was present in the normal population and to assess the frequency of the alteration in the population. Restriction enzyme digests of hMSH2 exon 1 PCR product (obtained as in 2.8.1.1) contained 10 $\mu$ l PCR product, 10U *Bcn*1 and 1x buffer in a final volume of 20 $\mu$ l and were carried out at 37°C for at least 2 hours. This was followed by electrophoresis on a 20% polyacrylamide gel. 10ml gel was prepared by mixing 5ml of 40% 29:1 acrylamide:bis acrylamide with 1ml 10xTBE and made to 10ml with DDW. Just before pouring, 20 $\mu$ l 10% APS and 5 $\mu$ l TEMED were added to the gel solution, mixed and poured between 6x10cm glass plates separated by 0.8mm spacers and combs (BioRad Mini-Protean Gel System). The gel was allowed 30 minutes to polymerise, after which the wells were rinsed with 1xTBE and the samples, mixed with 0.5x volume loading dye, were loaded into the wells. Electrophoresis was carried out in 1xTBE at 100V until the xylene cyanol had migrated to the bottom of the gel. The gel was then removed, stained for 15 minutes in 50 $\mu$ g/ml ethidium bromide in 1xTBE and visualised under UV. This work was carried out by Mr R. Morris.



10x <i>BcnI</i> buffer:	Loading dye: see 2.4	TBE: see 2.4
100mM Tris-HCl (pH7.5)		
100mM MgCl <sub>2</sub>		
500mM NaCl		
1mg/ml BSA		

## 2.8.4 Heteroduplex analysis of hMSH2 PCR products

### *2.8.4.1 Preparation of PCR samples*

PCR reactions were carried out as above (section 2.8.1). PCR product was heated to 95°C to denature double-stranded DNA and then cooled slowly to 37°C over 30 minutes, allowing reannealing of single strands. Where mutant alleles are present, these form heteroduplexes with normal alleles. Since heteroduplex molecules tend to be retarded in comparison with homoduplexes on running through polyacrylamide gel, they are readily detectable by electrophoresis. Cooled samples were prepared for electrophoresis by adding 4µl to 2µl loading dye (see 2.4).

### *2.8.4.2 Electrophoresis*

35x50cm glass plates for vertical gel electrophoresis were used with 0.4mm spacers and square or shark's tooth combs. Both were pre-treated: the first was wiped with 3ml Gel Slick, allowed to dry for 5 minutes then wiped with 70% ethanol. The second was treated with 20µl gamma-methacryloxypropyl-trimethoxysilane in 5ml distilled water (pH 3.5 with acetic acid) in order to attach the gel to the plate for staining and dried for ten minutes before wiping four times with 95% ethanol. A 1xMDE gel was prepared by addition of 240µl 10% ammonium persulphate and 24µl TEMED to 60ml gel mix. The gel was poured and allowed to set for 1 hour before use. 5µl PCR product and loading dye prepared as above was loaded onto the gel along with 0.5µg of a molecular weight marker, Marker V (Boehringer Mannheim Ltd), and a control sample known to separate into heteroduplex and homoduplex bands. Electrophoresis was carried out at 0.71 to 0.8kV for 16 hours in 0.6x TBE running buffer. This slow running of the gel ensured that it remained cool.

MDE gel mix:	10xTBE:
30ml MDE	see 2.4
3.6ml 10xTBE	
9g urea	
DDW to 60ml	

#### **2.8.4.3 Silver staining of heteroduplex gels**

Gels prepared with MDE modified acrylamide, such as those used for heteroduplex analysis, were found to dissociate from bind silane-treated plates on addition of the acetic acid solution fixative stage of a standard silver staining protocol (section 2.7.2.3). Therefore, heteroduplex gels were stained using a different silver staining protocol. Minor modifications to this method resulted in high quality silver staining.

The entire protocol was carried out on a gently rocking platform using Ultrapure water (UDDW). The glass plates were separated and the gel soaked for 10 minutes in 10% ethanol followed by 10 minutes in 1% nitric acid. It was then rinsed for 5 minutes in UDDW and placed in staining solution for 20 minutes. After rinsing for 10 seconds with UDDW, the gel was placed in developer solution until a brown precipitate appeared, when the developer was replaced with fresh. When bands appeared, the reaction was stopped with 0.1M citric acid for 10 minutes, the gel rinsed in UDDW for a further 5 minutes and then interpreted and photographed on a light box. For re-use, plates were soaked in 5M sodium hydroxide for one hour and cleaned with detergent and 70% ethanol. Spent silver nitrate was treated with sodium chloride to precipitate the silver before disposal.

Staining solution:

2.04g silver nitrate in 1l UDDW

Developer solution:

160g sodium carbonate (decahydrate)

1ml 37% formaldehyde

in 2l UDDW

## **2.9 Sequencing of mutations**

### **2.9.1 Strategy**

All tumours showing aberrantly migrating bands by SSCP or heteroduplex analysis of hMSH2 were sequenced along with their corresponding normal DNA using the dideoxy chain termination method (Sequenase version 2.0, United States Biochemicals). Exon 1 was sequenced directly from PCR product. Exons 2, 10, 12, 13, 15 and 16 were not amenable to direct sequencing and so were first made blunt-ended, cloned into a vector and sequenced as either single or pooled colonies. For exons 3 and 5, biotinylated primers were available, allowing sequencing to be carried out after separation of the DNA into single strands using Dynabeads (Dynal Ltd). Further sequencing was carried out using high-fidelity polymerase where single *Taq* polymerase-amplified clones had been analysed, as the rate of *Taq* polymerase-induced errors was found to be high.

## 2.9.2 Cloning and sequencing of blunt-ended PCR products

### *2.9.2.1 PCR reactions and creation of blunt-ended DNA*

PCR was carried out as described in section 2.8.1.1 to produce 200µl of each sample to be sequenced. Surplus reaction reagents were removed using a fine sephadex G50 Quick Spin column (Boehringer-Mannheim Ltd). The column was first inverted to resuspend the sephadex and then spun at 6000 x g for two minutes to dry. 100µl PCR product was added to the top of the column and it was spun at 6000 x g for 4 minutes and the drops collected. This was repeated with the remaining 100µl product. The cleaned PCR product was precipitated in 20µl 7.5M ammonium acetate and 400µl cold absolute ethanol (-20°C) at -70°C for 30 min, spun at 10000 x g for 15 min at 4°C, washed in 400µl cold 70% ethanol and spun again at 4°C for 15 minutes. The pellet was drained, vacuum dried for 15 min and resuspended in 16.5µl DDW. *Taq* polymerase-generated products were then treated with the Klenow fragment of DNA polymerase I to produce blunt-ended fragments. PCR product was incubated at room temperature with 5U Klenow in a final concentration of 1x buffer and 0.5mM dNTPS. The reaction was stopped after 30 minutes by adding 1µl 0.5mM EDTA pH8 and the DNA spun through a G50 Quick Spin column (Boehringer-Mannheim Ltd), as in 2.9.2.1, to remove unincorporated dNTPs. DNA was quantitated by electrophoresis of 5µl sample against 2µl quantitative DNA mass ladder on a 2% agarose gel containing as in 2.4 and checked under UV.

Klenow buffer:

Any restriction enzyme buffer can be used (eg *Pst*I buffer, see section 2.8.1.4)

### *2.9.2.2 Preparation of vector*

2µg pGEM 7zf+ vector was linearised with 10U *Sma*I at 25°C overnight in 1x buffer and surplus reagents removed using a G50 Quick Spin column (Boehringer Mannheim Ltd) as in 2.9.2.1. Digestion was checked by electrophoresis of 500ng DNA on a 0.8% agarose gel containing 50ng/ml ethidium bromide and run in 1xTBE against 500ng uncut vector and λ DNA/HindIII molecular weight marker (Boehringer-Mannheim Ltd). The gel was checked on a UV box.

10x <i>SmaI</i> buffer:	1x TBE:
330mM Tris acetate	see 2.4
100mM magnesium acetate	
660mM potassium acetate	
50mM dithiothreitol	
pH 7.9 at 37°C	

### 2.9.2.3 Ligation reactions

For each sample to be sequenced 2 ligation reactions were set up, one with a 3:1 and one with a 10:1 molar ratio of insert:vector. Reactions consisted of 100ng vector, 30ng or 100ng blunt-ended PCR product, 1x buffer and 5U T4 ligase in 20µl volume. A negative control, containing everything except ligase, was also included. Ligations were carried out at 16°C overnight.

A positive (no insert) and a negative (no insert or ligase) control was also included.

5xT4 Ligase buffer:  
 250mM Tris-HCl pH7.6  
 50mM magnesium chloride  
 5mM ATP  
 5mM dithiothreitol  
 25% (w/v) polyethylene glycol-8000

### 2.9.2.4 Transformation of competent cells

Transformations were carried out in DH5α competent *E coli* cells. These cells undergo α-complementation of the β-galactosidase gene when transformed with an appropriate plasmid and can therefore be used for blue/white colony screening when grown in the presence of X-gal.

Ligation reactions, including the positive and negative control, were diluted 1/5 with DDW and 1µl (about 1-2ng) added to 20µl competent cells (thawed on ice) and gently mixed. After 30 minutes on ice, the cells were heat shocked at 42°C for 40 seconds then placed immediately on ice again. 80µl SOC medium was added to each and the tubes were shaken at 37°C at 300rpm for one hour. The entire cell suspension was then plated onto a 50µg/ml L-Amp agarose plate spread with 800µg X-Gal and 800µg IPTG in 1ml L-broth for blue-white screening. Plates were inverted and incubated overnight at 37°C. Remaining competent cells were refrozen at -70°C by first placing in a methanol/dry ice bath for five minutes. Cells were not used if they had been subject to more than one freeze-thaw cycle.

SOC medium:	L-amp plates:	L-broth:
20mg/ml bactotryptone	L-broth containing:	10g/l Bacto-tryptone
5mg/ml yeast extract	100µg/ml ampicillin	10g/l NaCl
10mM NaCl	12g/l Bacto-agar	5g/l yeast extract
2.5mM KCl		Autoclaved before use
autoclaved and cooled before adding:		
10mM MgCl <sub>2</sub> .6H <sub>2</sub> O (filter sterilised)		
10mM MgSO <sub>4</sub> .7H <sub>2</sub> O (filter sterilised)		
20mM glucose (filter-sterilised)		
pH7.0		

TE: see 2.4

#### 2.9.2.5 Minipreparations

Initially, all samples were grown as minipreparations as pooled clones by picking between 20 and 100 separate large white colonies (containing insert) from each plate using sterile pipette tips. All colonies were put into 10ml L-broth containing 50µg/ml ampicillin and incubated at 37°C, shaking at 300rpm, overnight. It later became necessary to sequence single colonies (see section 2.9.1), and these were grown up in exactly the same way but adding only one colony to each miniprep. Colonies were only used if the concurrent control plates appeared correct, ie positive control showed blue colonies and negative control showed no (or very few) colonies.

Extraction of DNA was carried out using the Wizard Miniprep kit (Promega Ltd). 3ml broth was spun at 10000 x g to pellet cells. The pellet was resuspended in 200µl resuspension solution and 200µl cell lysis solution added and the tube inverted until the solution cleared. 200µl neutralization solution was then added and the tube inverted to mix. The solution was then spun 10000 x g for 5 minutes and the supernatant mixed with 1ml Wizard Miniprep DNA Purification Resin and mixed. This was poured into the barrel of a 5ml syringe attached to a Wizard Minicolumn, placed into a vacuum manifold and a vacuum applied. The vacuum was broken and 2ml column wash added and the vacuum reapplied. After emptying the tube, the column was dried by applying the vacuum for a further 2 minutes. The syringe barrel was then removed and the column spun at 10000 x g for 20 seconds to remove any residual column wash. Fifty microlitres of TE was added and after 1 minute the column spun for 20 seconds into a fresh tube to collect eluted DNA

L-broth: see 2.9.2.4	Resuspension solution: 50mM Tris-HCl pH 7.5 10mM EDTA 100µg/ml RNase A	Cell lysis solution: 200mM NaOH 1% SDS	Neutralization solution: 1.32M potassium acetate, pH4.8
Wizard Minipreps DNA Purification	Column wash solution: 200mM NaCl		TE: See 2.3.1
Resin: constituents not given by manufacturer	20mM Tris-HCl pH7.5 5mM EDTA diluted with 55ml 95% ethanol prior to use		

#### 2.9.2.6 Sequencing reactions

Sequencing of cloned PCR fragments was carried out according to the protocol provided with the Sequenase Version 2.0 kit (Amersham International plc). Double-stranded plasmid DNA containing insert, obtained as above, was denatured by incubating at 37°C for 30 minutes in 0.1 volume denaturing solution. This was neutralised with 0.1 volume 3M sodium acetate, pH4.5-5.5, and precipitated with 4x volume ethanol. After 15 minutes at -70°C, the DNA was centrifuged at 4°C for 15 minutes, washed with 4x volume 70% ethanol, centrifuged again and vacuum dried. The pellet was resuspended in 7µl DDW, and to it was added 50pg of appropriate primer in 1µl volume and 2µl Sequenase buffer. This was mixed and heated to 65°C for 2 minutes then allowed to cool to <35°C in a waterbath over 15-30 minutes. It was then spun briefly and placed on ice. To it was added 1µl 0.1M DTT, 2µl labelling mix (diluted 1/5 in DDW) 3.2U Sequenase in 2µl dilution buffer and 5µCi <sup>35</sup>SdATP. 2.5µl of each termination mix was warmed to 37°C and 3.5µl of the the above DNA solution added and mixed thoroughly. After 5 minutes the reaction was stopped by adding 4µl stop solution and the reactions stored at -20°C for up to one week before use.

The primer used for sequencing was either the T7 promoter for sequencing single clones, or the appropriate PCR primer, either upstream or downstream, for pooled clones.



Denaturing solution:	5 x Sequenase buffer:	Labelling mix	Termination mix:
2M NaOH	200mM Tris-HCl pH 7.5	(dGTP):	50mM NaCl
2mM EDTA	100mM MgCl <sub>2</sub>	7.5μM dGTP	80μM each dNTP
	250mM NaCl	7.5μM dTTP	8μM of one of
		7.5μM dCTP	ddGTP, ddATP,
			ddTTP or ddCTP

Dilution buffer:	Stop solution:
10mM Tris.HCl pH 7.5	see 2.7.3.2
5mM dithiothreitol	
0.5mg/ml BSA	

### 2.9.2.7 Sequencing gels

Sequencing was carried out on BRL S2 sequencing apparatus using 0.4mm spacers and sharks-tooth combs. Plates were cleaned with cream cleaner, washed thoroughly and wiped with 70% ethanol. One plate was treated with gel slick as in section 2.7.2.1. 60ml gel stock solution was polymerised by adding 60μl 25% ammonium persulphate and 60μl TEMED immediately before pouring. The gel was allowed to polymerise horizontally for 1 hour with inverted combs and then pre-run without combs, after rinsing the well thoroughly with 1xTBE, at 90W for 30-60 minutes in 1xTBE until it reached 50°C. Combs were then replaced to form wells, which were again rinsed thoroughly. Samples were heated to 75°C for 3 min prior to loading and run at 70W until the bromophenol blue marker reached the bottom of the gel.

The gel was fixed twice in 10% acetic acid 10% methanol for 5 minutes and transferred onto 3MM paper. It was dried for 2 hours on vacuum gel dryer and exposed to autoradiography film overnight.

Stock gel solution:	10xTBE:
75ml 19:1 acrylamide:bis acrylamide 'Instagel' (Severn Biotech Ltd)	See 2.4
250g 500ml urea (7M)	
50ml 10x TBE	
175ml DDW	
stored protected from light at 4°C	

### 2.9.3 Sequencing using high-fidelity polymerase

#### **2.9.3.1 Overcoming the problem of *Taq* polymerase-generated sequence errors in single-clone sequencing**

The error rate of *Taq* polymerase is high, estimated to range from one error per 400 bases to one per 4-5000 under more optimum conditions (Saiki *et al.*, 1988; Innis *et al.*, 1988). Therefore, the method of choice to produce the most reliable data relies on pooling a large number of cloned PCR products. This reduces the likelihood that a *Taq*-generated error will be seen in the subsequent DNA sequence, since only errors which occurred at an early stage of amplification will produce sufficient copies to be detected amid large numbers of normal copies. However, it requires sequencing reactions to be primed by an oligonucleotide specific to the PCR-generated fragment rather than one specific to the plasmid, since orientation of integrated blunt-ended cloned products within the plasmid is random. Primers designed from such a sequence are not always suitable for sequencing, presumably because they form inappropriate secondary structure. Consequently, whilst sequencing of pooled clones produced very clear results for some exons of hMSH2 it was not always successful, giving many 'stops' across all four lanes of sequencing reaction in some exons. In order to employ a plasmid-specific primer, single clones had to be sequenced but, not surprisingly, when this was attempted using cloned *Taq*-gene fragments, many false mutations were detected (ie apparent mutations could not be confirmed on multiple repetitions of the reactions). For exons which could not be sequenced using pooled clones, PCR fragments were generated using a high-fidelity polymerase, *rTth* (Perkin-Elmer Ltd), which has proofreading activity. This resulted in high quality sequence with few spurious results.

#### **2.9.3.2 High Fidelity PCR of hMSH2**

*rTth* polymerase is a high fidelity proofreading DNA polymerase which produces fragments with adenine overhangs similar to *Taq* polymerase-generated fragments. Although PCR yields tend to be significantly lower than with *Taq*, similar reaction conditions produce perfectly adequate results. PCR reactions were carried out in 50µl volumes containing 100ng DNA, 1mM magnesium acetate, 200µM each dNTP, 50µM each primer and 1x buffer. Reactions were 'hot started' by adding 2U *rTth* DNA polymerase, XL as in the manner described in section 2.8.1.1. Cycling conditions were the same as in section 2.8.1.1 and Table 3.

*rTth* buffer:

tricine, potassium acetate, glycerol, DMSO  
(concentrations not given by manufacturer)

### 2.9.3.3 Cloning into pCRII

pCRII vector (part of the TA cloning kit, Invitrogen) became available at this stage and was used in preference pGEM7ZF+ as it is quicker and more efficient. It removes the need for blunt-end cloning, since the vector, pCRII, is linear and has thymine overhangs, making it very easy to ligate to DNA fragments, which have adenine overhangs. *rTth* polymerase was used in conjunction with this vector to produce cloned sequences.

The PCR reactions carried out with *rTth* polymerase were used as soon as possible after completion, always on the same day, in order to maximise ligation efficiency, since the adenine overhangs of the PCR product are displaced over a period of several hours. Ten microlitres of PCR product was quantified by electrophoresis on a 1.5% agarose gel containing 50ng/ml ethidium bromide by running against 2 $\mu$ l DNA mass ladder (Life Technologies Ltd). A 1:1 molar ratio of vector:insert ligation reaction was set up consisting of 2 $\mu$ l 10x ligation buffer, 2 $\mu$ l (50ng) pCRII vector, 1 $\mu$ l (4 units) T4 DNA ligase and 5ng PCR product (up to 2.5 $\mu$ l) in a final volume of 10 $\mu$ l. If PCR reaction yield was lower than 2ng/ $\mu$ l, 3 $\mu$ l was added and amount of vector reduced accordingly to retain a 1:1 molar ratio. All components were kept on ice as far as possible. Reactions were incubated for 24 hours at 14°C.

10x Ligation buffer:

60mM Tris-HCl, pH7.5

60mM magnesium chloride

50mM sodium chloride

1mg/ml bovine serum albumin

70mM  $\beta$ -mercaptoethanol

1mM ATP

20mM dithiothreitol

10mM spermidine

### 2.9.3.4 Transformation of competent cells

Fifty microlitres of One Shot Inv $\alpha$ F<sup>+</sup> competent cells (part of the TA cloning kit) were thawed on ice and mixed gently by stirring with a pipette tip. Two microlitres of 0.5M  $\beta$ -mercaptoethanol, followed by 2 $\mu$ l ligation mix (obtained as above), were added, stirring gently after each. After a 30 minute incubation on ice, cells were heat-shocked for 30 seconds at 42°C and placed on ice for a further 2 minutes. To this was added 450 $\mu$ l SOC medium and the vials shaken at 225rpm and 37°C for 1 hour. The cells were then placed on ice before spreading a 50 $\mu$ l and a 200 $\mu$ l aliquot L-amp plates which had previously been spread with

800µg X-Gal in 1ml L-broth. Plates were incubated overnight at 37°C and then stored at 4°C.

SOC medium, L-broth and L-amp plates:

see 2.9.2.4

### **2.9.3.5 Minipreparations**

Minipreparations were carried out as in 2.9.2.5

### **2.9.3.6 Sequencing reactions and electrophoresis**

Sequencing reactions and gels were as in 2.9.2.6-2.9.2.7, using only the T7 promoter primer. All apparent mutations were confirmed by their presence in at least one other clone. Generally, 6-8 clones were sequenced per sample.

## **2.9.4 Sequencing using Dynabeads**

### **2.9.4.1 Preparation of single-stranded DNA**

PCR reactions were carried out as described in 2.8.1.1, substituting the reverse primer for one biotinylated at its 5' end. 100µl product was generated and spun through a G50 Quick Spin column (Boehringer Mannheim Ltd) (see 2.9.2.1) to remove excess reagents.

It was then added to an equal volume of wash buffer and 15µl streptavidin-coated Dynabeads (Dynal Ltd), left for 10 minutes and placed next to a magnet to separate the beads. The supernatant was removed and the beads washed with 100µl wash buffer and the supernatant removed by magnetic separation. The beads were resuspended in 5µl fresh 0.15M NaOH and incubated at room temperature for 10 min, placed next to magnet and the supernatant, containing unbiotinylated DNA strand, removed and stored in cold ethanol. This step was repeated, the beads washed with 100µl wash buffer followed by 100µl TE and the beads resuspended in 10µl DDW. Five microlitres were used for each sequencing reaction.

Wash buffer:	TE:
10mM Tris pH7.5	See 2.8.1.4
1mM EDTA	
2M NaCl	

### **2.9.4.2 Sequencing single-stranded DNA prepared using Dynabeads**

For each 5µl sample to be sequenced, 1µl DMSO, 10ng of the appropriate upstream PCR primer and 2µl Sequenase buffer was added. The DNA was denatured at 95°C for 3 minutes, placed immediately on ice, briefly spun and replaced on ice. Dideoxy termination mixes (ddGTP, ddATP, ddTTP, ddCTP) were prepared by adding 10% DMSO to each. For

each sequencing reaction, 2.5µl of each mix was placed at 37°C and allowed to prewarm for at least 5 minutes.

To each DNA sample, 1µl 0.1M dithiothreitol, 2µl dGTP labelling mix diluted 1/10-1/20 with DDW, 0.5µl <sup>35</sup>S dATP (10µCi), 2µl Sequenase 2.0 diluted in Sequenase dilution buffer to 13U/6µl was added sequentially and mixed. 3.5µl of this mix was added to the termination mixes at 37°C, mixing well. After 5 minutes, the reaction was stopped by adding 4µl stop solution.

Stop solution:

see 2.7.3.2

Other reagents:

see 2.9.2.6

#### ***2.9.4.4 Sequencing gels***

Sequencing gels were prepared and run as in section 2.9.2.7.

#### **2.9.5 Cycle sequencing of PCR products**

Direct sequencing of PCR products was carried out using the ThermoSequenase radio labelled terminator cycle sequencing kit (Amersham International plc). This is a method of cycle sequencing which utilises radio-labelled ddNTP terminators in conjunction with a thermostable DNA polymerase to produce high quality sequence which is largely free of background.

##### ***2.9.5.1 Pretreatment of PCR DNA samples***

Before sequencing reactions can be carried out, PCR-generated DNA must be pretreated with exonuclease I and shrimp alkaline phosphatase (SAP) to remove single-stranded oligonucleotides, single-stranded PCR-generated DNA and free dNTPs. To do this, 1µl each enzyme (10U exonuclease I and 2U SAP) was added to 5µl PCR product and incubated at 37°C for 15 minutes. The enzymes were then inactivated by heating to 80°C for 15 minutes. Additionally, an aliquot of untreated DNA sample was quantitated by agarose gel electrophoresis against a quantitative marker as in 2.4.

##### ***2.9.5.2 Sequencing reactions***

2µl dGTP termination master mix was mixed with 0.5µl of [ $\alpha$ -<sup>33</sup>P]ddNTP to produce a termination mix for each ddNTP. A reaction mixture was made for each test DNA sample by mixing 50ng (25fmol) treated DNA with 0.5pmol sequence-specific primer (forward-strand primers as described in Table 3) and 2µl reaction buffer in a final volume of 20µl. 4.5µl reaction mixture was transferred to each termination tube, mixed and overlaid with one drop of paraffin oil. The tubes were placed on a thermal cycling block and subjected to 30 cycles of 95°C for 30s, 57°C for 30s and 72°C for 1 minute. 4µl stop solution was added to each

tube and the samples heated to 70°C for 2 minutes before loading 3µl per lane onto a denaturing polyacrylamide gel.

Reaction buffer:	dGTP termination master mix:	Stop solution:
260mM Tris HCl, pH9.5	7.5µM dGTP	see 2.7.3.2
65mM MgCl <sub>2</sub>	7.5µM dATP	
	7.5µM dTTP	
	7.5µM dCTP	

### **2.9.5.3 Gel electrophoresis**

Gel electrophoresis and autoradiography were carried out as in 2.9.2.7, except that glycerol-tolerant buffer was substituted for TBE buffer.

20x glycerol tolerant buffer:

216g Tris base

72g taurine

4g Na<sub>2</sub>EDTA 2H<sub>2</sub>O

made to 1litre with DDW

### **2.9.4.4 Elimination of compressions using dITP**

Where compressions (uneven band spacing) caused difficulties in reading a sequence, reactions were repeated substituting dITP termination master mix for dGTP. Reactions were carried out as above, except that the termination temperature was reduced from 72°C to 60°C and the time at this temperature increased to 5 minutes.

dITP termination master mix:

7.5µM dITP

7.5µM dATP

7.5µM dTTP

7.5µM dCTP

## **2.10 Assay for the effect of hMSH2 polymorphisms on transcription of the gene**

### **2.10.1 Strategy**

It was postulated that the intronic mutations that were detected within hMSH2 might affect mRNA splicing by causing aberrant splicing (most likely skipping of the adjacent exon in the case of mutation affecting a splice donor site or failure to excise an intron in the case of



mutation affecting a splice acceptor site). Alternatively, they could result in reduced levels of transcript. In order to investigate this possibility, RNA was extracted from 8 xenografts, 4 of which were known to have an intronic mutation in their primary tumour (all 4 in intron 5 and one in intron 1) and 4 of which were non-mutated controls. RNA was not available from primary tumour. cDNA was produced by reverse transcription and two assays were carried out. In the first procedure, PCR primers were designed to amplify three regions: across exon 2 from exons 1 and 3, across exon 5 from exons 4 and 6 and across this whole region from exons 1 and 6. Amplification of these regions was used to look for evidence of aberrant splice sequences. Secondly, since insufficient tumour material was available for extraction of RNA in adequate quantities for Northern blotting, a quantitative PCR-based assay was used to ascertain the level of transcript present in mutated versus normal samples.

## 2.10.2 Extraction of RNA from frozen tissue

### *2.10.2.1 TRIzol method*

Extraction of RNA from frozen tissue was carried out using two different methods. The first utilized TRIzol (Life Technologies Ltd), a mono-phasic solution of phenol and guanidine isothiocyanate which isolates total RNA from cells. Tissue samples were handled in a class one flow hood using appropriate measures to avoid RNase contamination: all glassware was sterilised at 180°C before use, plastic was rinsed with diethyl pyrocarbonate (DEPC)-treated water, tips and tubes were from new unopened packets and double gloves were worn when handling all materials.

A tissue sample at least 5mm<sup>3</sup> was taken from the -70°C freezer and stored on dry ice until needed. The sample was placed into 1ml TRIzol reagent in a 1.5ml tube and macerated using a small plastic stick. After 5 minutes at room temperature, 0.2ml chloroform was added, the tube vigorously shaken for 15 seconds and then left at room temperature for a further 3 minutes. It was spun at 12000g for 15 minutes at 4°C and the upper aqueous layer which appeared after centrifugation was removed to a clean tube. RNA was precipitated by adding 0.5ml isopropanol, incubating for 10 minutes at room temperature and then spinning at 10000 x g for 10 minutes at 4°C. The RNA pellet was washed by adding 1ml 75% ethanol, vortexing and spinning at 7500 x g for 5 minutes at 4°C. The supernatant was removed and the tube left inverted on a clean paper towel for 10 minutes until the pellet was almost dry. It was then dissolved in 20µl DEPC-treated DDW and incubated at 60°C for 10 minutes. RNA was quantitated on a spectrophotometer at A<sub>260</sub> and stored at -70°C.

DEPC-treated water:

DEPC in DDW

Shaken and left for 24 hours to destroy RNases, then autoclaved to destroy DEPC.

#### ***2.10.2.2 QuickPrep Total RNA Extraction kit method***

The QuickPrep Total RNA Extraction kit (Pharmacia Biotech) is an extraction procedure in which guanidinium thiocyanate (GTC) treatment dissociates cellular components and inactivate endogenous RNase, and RNA is selectively precipitated by centrifugation in caesium trifluoroacetate (CsTFA).

Precautions for avoidance of RNase contamination were followed as above

(2.10.2.1). A 5mm<sup>3</sup> tissue sample, stored at -70°C then on dry ice immediately before use, was placed into a 1.5ml tube and homogenised in 150µl extraction buffer and 3µl β-mercaptoethanol (β-ME) using a small plastic stick. To this was added 350µl lithium chloride solution, the solution mixed thoroughly and placed on ice. 500µl CsTFA was added, the sample mixed by vortexing, and then placed on ice for 10 minutes. RNA was pelleted by centrifuging at 10000 x g at 4°C, the supernatant aspirated, and the tube placed on ice prior to washing the pellet with 75µl extraction buffer, 175µl lithium chloride solution and 250µl CsTFA. The sample was vigorously vortexed for 5 pulses to remove any remaining protein and DNA contaminants from the pellet and tube, and then centrifuged at 10000 x g for 10 minutes at 4°C. The supernatant was aspirated, the tube placed on ice and 1ml 70% ethanol added. After vortexing for 5 pulses, the samples was again spun at 10000 x g for 5 minutes at 4°C, the ethanol removed and the pellet air dried for 10-15 minutes. RNA was dissolved in 100µl DEPC-treated DDW (see 2.10.2.1) on ice for 30 minutes, vortexed, and then heated to 65°C for 10 minutes with occasional pipetting to aid dissolution of the pellet. Quantitation and storage was as in 2.10.2.1.

#### **2.10.3 Preparation of first strand cDNA.**

First strand DNA synthesis was carried out using the Superscript Preamplification System with random hexamers (Life Technologies Ltd). 1µg RNA prepared as above was defrosted on ice and to it was added 500ng random hexamers. The mixture was heated to 70°C for 10 minutes and transferred to ice for 1 minute. To the tube was added 2µl PCR buffer, 2µl 25mM MgCl<sub>2</sub>, 1µl dNTP mix and 2µl 0.1M DTT. This was incubated at 25°C before adding 1µl (200U) Superscript II RT, mixing and incubating for a further 10 minutes at 25°C then for 50 minutes at 42°C. Reactions were terminated by heating to 70°C for 15 minutes and the tubes then chilled on ice. Finally, the newly-synthesised cDNA was incubated with 1µl (2U) *E coli* DNase-free RNase H for 20 minutes at 37°C to remove original RNA.

PCR buffer:

see 2.7.1

## 2.10.4 Assay 1

### 2.10.4.1 Polymerase chain reaction of hMSH2 cDNA

PCR of hMSH2 cDNA was carried out using the primers shown in Table 4. Reactions were 'hot started' as in section 2.8.1.1 and reagents were identical to those described in section 2.8.1.1, with substitution of 2µl cDNA product for genomic template. Cycling conditions were 30 rounds of 30 seconds at 94°C, 30 seconds at 50°C (exons 1-3) or 54°C (exons 4-6) and 1 minute at 72°C. In addition, the forward primer for exon 2 amplification and the reverse primer for exon 5 amplification (Table 4) were used to amplify a sequence from exon 1 to exon 6. For this reaction, annealing temperature was 56°C and predicted normal product was 1072bp.

**Table 4**

**Primer sequences and predicted PCR product size for hMSH2 cDNA amplification.**

Exons amplified	Predicted size (bp)		Primer sequence (5' to 3')	PCR annealing temperature
	Normal	Predicted aberrant splicing product		
2	610	455	GCGCATTTTCTTCAACCAGG GAGGAGAGCCTCAAGATTG	56°C
5	324	510	TATCAGGACCTCAACCGG CCACTGGTTAACAAGTCTTT	52°C

### 2.10.4.2 Detection of amplified cDNA sequence by polyacrylamide gel electrophoresis

PCR-amplified DNA was electrophoresed on a 2% agarose gel (as in section 2.4) and the fragment sizes visualised and photographed under UV light. For more accurate determination of band sizes, the fragments were then electrophoresed on a 5% (exon 2) and 8% (exon 5) acrylamide minigel. This was as in 2.8.3, except that 1.25ml or 2ml, respectively, of 40% 29:1 acrylamide:bis were used.

## 2.10.5 Assay 2

### **2.10.5.1 Quantitative PCR of hMSH2**

PCR has been shown to be a reliable method for quantitation of mRNA (Wang *et al.*, 1989). In the assay used here, the relative amount of hMSH2 mRNA transcript present in tumours bearing sequence alterations in introns 1 and 5 and those with no sequence change was compared using a quantitative PCR assay (Wei *et al.*, 1995). The amount of hMSH2 transcript was measured in relation to  $\beta$  actin transcript, which was co-amplified as an internal standard, and quantitated against cDNA from normal colonic tissue.

cDNA (produced in step 2.10.3) was amplified using the primers shown in Table 5. Because the amount of  $\beta$  actin transcript is many times greater than that of hMSH2, the method of Wei *et al.* recommends addition of  $\beta$  actin primers after 11 cycles, in order to ensure that the reaction remains in log phase. This was found to work for cDNA generated from TRIzol-extracted RNA, but had to be modified to addition after 7 cycles for cDNA derived from Pharmacia-extracted RNA because levels of  $\beta$  actin product, though consistent, were generally lower. This was presumably due to the carry-over of very low levels of extraction reagents into cDNA, which is only likely to be problematic when primers are insufficiently pure (Dr S. Bader, personal communication). Resynthesis of higher quality oligonucleotides would probably eliminate this difficulty. Despite this minor problem, cDNA produced from RNA extracted by the Pharmacia kit method was found to produce more consistent results on quantitative PCR analysis, and therefore only RNA extracted by this method was used for quantitation.

PCR reactions consisted of 1.8 $\mu$ M each hMSH2 primer, 2.5U *Taq* polymerase, 1.2mM MgCl<sub>2</sub>, 1x PCR buffer, 0.1mM of each dNTP and 1 $\mu$ l cDNA in a final volume of 45  $\mu$ l, overlaid with one drop of paraffin oil. Cycling was carried out for 11 cycles of 95°C for 30 seconds, 59°C for 30 seconds and 72°C for 1 minute. After this, 45 $\mu$ M of each  $\beta$  actin primer was added to make a final volume of 50 $\mu$ l, and a further 19 cycles carried out at the same temperatures. Products were visualised on a 2% agarose gel as in 2.4. Quantitation was carried out using the BioRad Gel Doc system and Molecular Analyst software.

Reactions were carried out in triplicate.

PCR buffer:

Sec 2.7.1

**Table 5**  
**Primer sequences for quantitative hMSH2 cDNA assay.**

Amplified sequence	Primer sequence (5' to 3')	Product size (bp)
hMSH2	GTCGGCTTCGTGCGCTTCTTT TCTCTGGCCATCAACTGCGGA	429
β actin	ACACTGTGCCCATCTACGAGG AGGGGCCGGACTCGTCATACT	621

**2.11 Mutation analysis of the p53 gene**

Mutation analysis of the p53 gene was performed on some tumours with low or negative immunohistochemical p53 staining in order to exclude the possible presence of mutation not detectable by IHC (see Chapter 5, Results). Analysis was carried out using single-stranded conformational polymorphism analysis (SSCP) of exons 4-8, in which 90% of all mutations are located (Levine *et al.*, 1991). Four samples with high levels of p53 staining had already been assessed in this manner by Ms J. Scheler and Ms K. Cripps, and found to harbour mutations within this region of the gene. Analysis of a further 20 tumours which were either negative or showed a low level of staining by immunocytochemistry with anti-p53 antibodies was carried out. The four samples with known mutations were included as positive controls.

2.11.1 PCR of p53 exons 4-8

PCR was carried out in four separate reactions using the primers listed in Table 6. Reactions were carried out in 50µl volumes consisting of 100ng genomic DNA, 0.5µM each primer, 200µM each dNTP, 1.5mM MgCl<sub>2</sub> and 1 x buffer solution, overlaid with one drop of paraffin oil. Reactions were 'hot-started' with 1.25U thermostable *Taq* polymerase as in 2.8.1.1 and cycled 30 times at 94°C for 30 seconds, 58°C for 30 seconds and 72°C for 1 minute. Reactions were checked on a 2 % agarose gel as in 2.4 before SSCP analysis.

**Table 6**

**Primer sequences for amplification of p53.**

p53 exon	Primer sequence (5'-3')
5	TTCCTCTTCCTACAGTAGTC CCCAGCTGCTCACCATCG
6	CCTCACTGATTGCTCTTAGG AGTTGCAAACCAGACCTCAG
7	TGTGTTATCTCCTAGGTTGG TGGCAAGTGGCTCCTGAC
8	TCCTATCCTGAGTAGTGGT TCCTGCTTGCTTACCTCG

#### 2.11.2 SSCP analysis of p53

SSCP analysis of PCR fragments generated in 2.1.3.1 was carried out as in 2.8.1.2.

### **2.12 DNA fingerprinting**

DNA fingerprinting was undertaken in selected cases in order to confirm that DNA from xenograft tissue corresponded to that of the primary tumour and normal tissue from which it had been taken (see Chapter 4, Results). The probe chosen for DNA fingerprinting was 29C1, a sequence which recognises a highly polymorphic region of human telomeric DNA located in the pairing regions of the short arms of the sex chromosomes (Cooke *et al.*, 1985). Where insufficient DNA was available for Southern blotting, or in cases which produced equivocal results [as discussed below], a second fingerprinting method was used. This was a PCR-based protocol for analysis of a highly polymorphic *Alu* sequence within the 3' end of intron 1 of the p53 gene (Futreal *et al.*, 1991).

#### 2.12.1 Fingerprinting using p29C1

##### **2.12.1.1 Southern blotting**

###### *Electrophoresis and DNA transfer*

10µg genomic DNA prepared as in section 2.3.1 was digested overnight at 37°C with 30U EcoRI and 1x enzyme buffer in a 50µl volume. A 0.8% 20x20cm horizontal gel was prepared by boiling 2.4g agarose in 300ml 1xTBE buffer, adding 5µl 10mg/ml ethidium bromide and pouring when the gel had cooled to 55°C. 5µl bromophenol blue loading dye was



added to each sample and electrophoresis was carried out at 80mA in 1xTBE until the dye had migrated 20cm. 1µg of a DNA molecular weight marker 1kb ladder (Life Technologies Ltd) was run alongside the samples. The gel was photographed on a UV box alongside a fluorescent ruler for scale.

The gel was prepared for blotting by shaking gently for 45 minutes in 500ml sodium hydroxide solution, washing twice for 15 minutes in DDW and neutralised by shaking for a further 45 minutes in 500ml 1M Tris-Cl (pH5.5). It was then carefully transferred to the blotting apparatus which consisted of a 20x35cm 17MM paper wick placed over a perspex plate which was suspended over a tank of 20xSSC. The wick was soaked in 20xSSC and carefully rolled flat before placing the gel on top. A sheet of Hybond N+ nylon membrane cut to the size of the gel and clearly marked for orientation was soaked in DDW then 20xSSC, placed on top of the gel and rolled flat to remove bubbles. On top of this was placed a pile of paper towels, a perspex plate and a 1kg weight. Clingfilm was placed around the edges of the gel to prevent evaporation and aberrant buffer flow and the apparatus was left overnight for the DNA to transfer to the membrane. After this time the membrane was soaked for 45 minutes in neutralising solution and then dried and stored between two sheets of blotting paper until hybridisation.

10x EcoRI buffer:	Sodium hydroxide solution:	Neutralising solution:
500mM Tris HCl (pH8.0)	0.5M NaOH	0.5M Tris pH7.4
100mM MgCl <sub>2</sub>	1.5M NaCl	3M NaCl
500mM NaCl		
20xSSC:	Bromophenol blue loading	
88.2g sodium citrate	dye:	
175.3g NaCl	see 2.4	

#### *Preparation of probe*

The insert from plasmid p29C1 was cut out using the *Pst*I restriction enzyme. 20U enzyme were added to 4µg plasmid DNA and 1x *Pst*I buffer and the volume made to 100µl with DDW. Digestion was done for 3 hours at 37°C, after which 5µl bromophenol blue loading dye (see 2.4) was added to each sample. The insert was isolated by electrophoresis on a 1% agarose gel against the 1kb ladder molecular weight marker (Life Technologies Ltd). The gel was prepared by adding 0.5g low melting point agarose in 50ml 1xTBE, boiling and adding ethidium bromide to 1µl 50µg/ml before pouring into a 5x10cm mould at 55°C. After electrophoresis at 100V in 1xTBE to 8cm migration of the dye, the 1.7kb insert was visualised briefly under UV light (to minimise DNA damage), cut out of the gel using a scalpel and placed into a pre-weighed 1.5ml tube. DDW was added at 300µl per 100µg of gel

and the agarose melted by heating to 65°C for 5 minutes. Concentration was estimated from the original known quantity of plasmid digested. 25-50ng of insert was taken for labelling.

10xPst1 buffer:	10xTBE:
500mM Tris HCl (pH8.0)	See 2.4
100mM MgCl <sub>2</sub>	
500mM NaCl	

#### *Labelling using random primers*

Probe DNA was labelled using the Prime-It RmT kit (Stratagene Ltd). Briefly, 50ng of probe in agarose prepared as above was added to a single-use reaction tube containing random 9-mers, nucleotides, buffer and co-factors for use with  $\alpha^{32}\text{PdCTP}$  and the volume made to 42 $\mu\text{l}$  with DDW. This was boiled for 5 minutes and briefly spun to collect condensation. To this tube was added 5 $\mu\text{l}$  (50 $\mu\text{Ci}$ )  $\alpha^{32}\text{PdCTP}$  and 3 $\mu\text{l}$  Magenta DNA polymerase (4U/ $\mu\text{l}$ ), the contents mixed and incubated at 37°C for 30 minutes. After this time the reaction was terminated by the addition of 2 $\mu\text{l}$  stop mix (0.5M EDTA (pH8.0). Unincorporated oligonucleotides and isotope were removed by passing the probe through a drained G50 Sephadex nick column that had been rinsed with TE. The column was then cleaned by running 3ml TE through it. Probe was added along with 400 $\mu\text{l}$  TE and allowed to pass into the column bed. A further 400 $\mu\text{l}$  TE was added and the drops collected from the bottom of the column. An approximate measure of isotope incorporation into the probe was obtained by comparing the Geiger counter reading of the tube relative to the column.

TE:

See 2.3.1

#### *Hybridisation*

The nylon membrane was placed onto a square nylon mesh, rolled and placed into a hybridisation bottle. 20 ml hybridisation buffer was added the bottle placed on a rotating wheel in a 65°C oven for 4 hours to prehybridise. After this time, probe prepared as above was denatured by boiling for 3 minutes. The hybridisation buffer was poured out of the bottle, the denatured probe added and the buffer returned to the bottle for overnight incubation rotating at 65°C.

The filter was washed by pouring out the hybridisation buffer, replacing it with 50ml prewarmed 2x SSC and replacing the bottle in the oven at 65°C for 30 minutes. The same was done with 2x SSC/1% SDS and then 0.5x SSC/1% SDS. The filter was then removed from the bottle, placed into 0.1xSSC at room temperature and soaked for 30 minutes. It was wrapped in Clingfilm and placed next to a piece of autoradiography film in cassette containing

intensifying screens, placed at -70°C for two days and then developed using an automatic developing machine.

After autoradiography, the filter was stripped of hybridised probe by boiling for 20 minutes in 0.1x SSC/1% SDS, dried and stored.

Hybridisation buffer:

5g dextran sulphate dissolved in 25ml DDW at 65°C for 30 minutes, followed by addition of:

15ml 20xSSC

2.5ml 20% SDS

heated to 65°C.

Immediately before use, 10mg salmon sperm DNA denatured by boiling for 5 minutes before adding to above buffer at 65°C.

pH to 7, DDW to 1l

20xSSC:

3M NaCl

0.3M Trisodium citrate

pH7

## 2.12.2 Fingerprinting using an *Alu* polymorphism within the p53 gene

### **2.12.2.1 Polymerase chain reaction**

Polymerase chain reaction was carried out in two parts. Initially, specific primers were used to amplify p53 intron 1, then a nested primer was used to amplify the internal *Alu* repeat (Table 7). In the initial round of amplification, PCR was carried out in 50µl volume reactions containing 1 x reaction buffer, 1.5mM magnesium chloride, 200mM each dNTP, 0.5µM each primer and 100ng genomic DNA template overlaid with drop mineral oil. This was heated to 94°C for 5 minutes, cooled to 80°C and 1.25U Taq polymerase added to each reaction. Cycling reactions were 30 cycles of 30 seconds at 94°C, 30 seconds at 55°C and 30 seconds at 72°C.

The second round of amplification was carried out using as template 10µl of PCR product from the previous reaction. This was added to 0.8 x buffer, 150mM each dNTP, 0.7mM magnesium chloride, 2.5µM *Alu* primer and 1.25U Taq polymerase in a final volume of 50µl, overlaid with a drop of paraffin oil and amplification carried out for ten cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C 30 seconds. PCR product was checked on a 2% agarose gel as in 2.4 before final analysis on a silver-stained denaturing polyacrylamide gel by the same method as for microsatellite sequences (2.7.2). Allele sizes were 140, 130, 125, 120, 115 and 105 bases.

PCR reaction buffer:  
see 2.7.1

**Table 7**  
**Primer sequences for amplification of p53 intron 1 *Alu* region.**

	Primer sequence (5'-3')
1st reaction upstream primer	GCACTTTCCTCAACTCTACA
1st reaction downstream primer	AACAGCTCCTTTAATGGCAG
<i>Alu</i> primer	CCACTTGCACTCCAGCCTGGG

**2.13 Comparative Genomic Hybridisation**

Comparative Genomic Hybridisation (CGH) was carried out on a series of 29 xenografted human colorectal tumour samples and three primary tumours (see Chapter 5, Materials and Methods). CGH was also carried out on DNA from mouse tumours, and this is described in 2.14.

2.13.1 Preparation of Metaphase Chromosome Spreads

**2.13.1.1 Blood culture**

10 ml of fresh blood donated by a healthy male volunteer was collected in a Lithium heparin tube. RPMI medium (Dutch modification) was supplemented with 10% foetal bovine serum, phytohemagglutinin and L-glutamine and filtered through a 0.2µm filter. 10ml was placed into a flat-bottomed 25cm<sup>2</sup> plastic tissue culture flask. 0.75 ml blood was inoculated into this, swirled to mix, and cultures were incubated at 37°C for 72 hours. 1 hour before harvesting (at 71 hours), 100µl of 10µg/ml colcemid was added and the incubation continued. Cultures were transferred into 10ml centrifuge tubes and spun at 500 x g for 5 minutes to pellet cells. The supernatant was removed and the pellet resuspended in prewarmed (37°C) 0.075M KCl hypotonic solution and incubated in a 37°C waterbath for 10 minutes. The tubes were then spun again at the same speed, the supernatant removed and the pellet tapped to loosen it. Cells were fixed dropwise with gentle vortexing to a final volume of 5ml with fresh ice-cold fixative solution, placed at -20°C for one hour and spun at 500 x g for 5 minutes. Fixing and spinning was repeated twice to obtain a white pellet. Metaphase preparations were stored in 5ml fix at -20°C for up to a year.

RPMI medium (Dutch modification)	Fixative solution:
containing:	3 parts methanol to 1
10% foetal bovine serum (heat inactivated)	part glacial acetic
9µg/ml phytohemagglutinin-M	acid
2mM L-glutamine	
Filtered through 0.2µm filter before use.	

### 2.13.1.2 Preparation of Slides

Microscope slides were soaked overnight in 10% Decon detergent, rinsed for 1 hour in water and transferred to 100% ethanol containing a few drops of concentrated hydrochloric acid for storage. Metaphase preparation solutions were spun at 500 x g for 5 minutes to pellet cells, the supernatant discarded and the pellet resuspended in a few drops of fix to form a milky solution. Slides were polished with a soft lint-free cloth just before use and one drop of fixed chromosome preparation dropped directly on top. The position of the drop was marked with a diamond pencil. Slide quality was checked on a phase contrast microscope at x40 magnification. If necessary, spreading of chromosomes was facilitated by either increasing the dropping height, placing slides at -20°C for 2 minutes before use or by gently blowing on the drop of metaphase suspension.

Slides for CGH were stored at room temperature in a vacuum desiccator for 1-2 weeks. Each batch was tested for suitability for CGH before setting up large numbers. Slides for G-banding were aged by baking at 80°C for one hour and stored on the bench.

### 2.13.1.3 G-Banding of Metaphase Chromosome Spreads

Normal karyotype of the control samples was confirmed by G-banding. Slides for G-banding were left for at least 24 hours after baking before staining.

Slides were treated with fresh 0.005% trypsin in PBS for 20-40 seconds and then rinsed with cold PBS. They were placed into Giemsa staining solution for 5 minutes, rinsed with DDW and mounted with DPX under a clean cover slip. Karyotype analysis of 10 metaphases was carried out under x100 magnification.

PBS:	Giemsa staining solution:
See 2.6	Gurr's Giemsa in PBS pH 6.8

### 2.13.2 Extraction of control DNA

Control DNA for counter-hybridisation was obtained from lymphocytes obtained from two healthy male volunteers. 10ml peripheral blood was collected in 1% EDTA and DNA extracted as described in section 2.3.2. Metaphase chromosome preparations from

these individuals were made from a separate blood sample (section 2.13.1.1) and normal karyotype was confirmed by G-banding of metaphase chromosomes (see section 2.13.1.3).

### 2.13.3 Nick Translation Of Control And Test DNA

Normal male DNA and either primary tumour or xenograft DNA were labelled by incorporation of digoxigenin-11-dUTP (normal) or biotin-16-dUTP (test samples) by nick translation. The method of Kallioniemi *et al* (1992) suggests that optimal hybridisation can be achieved using labelled probes of 600-1000bp. In this study, it was found that fragment lengths of between 100 and 1000kb, assessed by running single-stranded DNA on an agarose gel, provided optimal hybridisation, with chromosomes appearing fuzzy if fragments were too small and poor hybridisation occurring if fragments were too large.

For nick translation, DNaseI (10u/ $\mu$ l) was diluted 1/12000 in ice-cold DDW. One 1.5ml microfuge tube per sample was placed on ice to cool and the following mixture added to each:

2 $\mu$ g of genomic DNA, either normal male or test sample  
100 $\mu$ M dATP  
100 $\mu$ M dGTP  
100 $\mu$ M dCTP  
80 $\mu$ M dTTP  
20 $\mu$ M digoxigenin-11-dUTP (control) or biotin-16-dUTP (test sample)  
4 $\mu$ l of 10x nick translation buffer  
DDW to 38 $\mu$ l  
1 $\mu$ l (10U) DNA polymerase I  
1 $\mu$ l diluted DNase I

The mixture was mixed gently, centrifuged and incubated at 16°C for 1 hour. It was then placed on ice and 10 $\mu$ l removed in order to check fragment size. This aliquot was boiled for 2 minutes, placed on ice for 2 minutes, pulse-centrifuged and then fragments separated by electrophoresis against a 1kb ladder molecular weight marker (Life Technologies Ltd) on a 1% agarose gel (1% agarose in 1xTBE, otherwise as in section 2.4) until the bromophenol blue had migrated 6cm. If any fragment was bigger than 1kb, a further 1 $\mu$ l DNaseI was added to the reaction tube and the 16°C incubation continued for 10-20 minutes. When fragments were between 100 and 1000 nucleotides long, the reaction was stopped by adding 2 $\mu$ l 0.2mM EDTA/1%SDS. The DNA was then cleaned through a Quick Spin column to remove unincorporated nucleotides (Boehringer Mannheim Ltd) (as in 2.9.2.1) and stored at -20°C until required.

10x nick translation buffer:  
 0.5M Tris pH 7.5  
 0.1M MgSO<sub>4</sub>,  
 1mM dithiothreitol  
 500µg/ml bovine serum albumin fraction V.

### 2.13.3.1 Verification of DNA labelling

At first, nick translation reactions were always checked to ensure that sufficient incorporation of labelled nucleotide had occurred. However, the reactions were found to be extremely reliable and were thereafter only verified if a new reagent was used. The assay was done using a colorimetric assay, coupling alkaline phosphatase (AP), via streptavidin or anti-digoxigenin antibody, to biotin or digoxigenin incorporated into DNA and detecting with an AP substrate. The substrates used, 5-bromo-4-chloro-3-indoyl phosphate (BCIP) and nitro blue tetrazolium (NBT), develop an intensely dark blue-purple insoluble reaction product on exposure to AP.

Nick translated DNA was quantitated against biotinylated PBR322 or digoxigenin-labelled lambda standards. One microlitre of neat, 1/10 and 1/100 dilutions of labelled DNA were dotted onto a small charged nitrocellulose membrane (Hybond N+), along with concentrations ranging from 1-100pg of the appropriate standard. The filter was allowed to dry and then shaken for 5 minutes in buffer 1 followed by 30 minutes in buffer 2. After a further rinse in buffer 1, the filter was incubated for 10 minutes in 10ml buffer 1 containing 10µl streptavidin alkaline phosphatase (to test biotin-labelled DNA) or anti-digoxigenin alkaline phosphatase (to test digoxigenin-labelled DNA), shaken for two 15 minute washes in buffer 1 and then for 5 minutes in buffer 3. Alkaline phosphatase substrate BCIP/NBT was obtained in kit form and prepared according the manufacturer's instructions (Dako Ltd). It was incubated with the filter in a sealed plastic bag in the dark until colour developed (up to 2 hours), the test samples compared with the standard, and concentration of labelled DNA estimated.

Buffer 1:	Buffer 2:	Buffer 3:
0.1M Tris	0.1M Tris	0.1M Tris pH9.5
0.15M NaCl	0.15M NaCl	
pH7.5	0.5% blocking agent (Amersham International plc), dissolved at 60°C for 60 minutes prior to incorporation into buffer 2.	



#### 2.13.4 Hybridisation

Several variations of the CGH technique were attempted in order to find optimal hybridisation conditions. The method found to work most consistently was a modification of the method of Kallioniemi *et al* (1992), including changes incorporated from the methods of Verma and Babu and from a method provided by Dr H.Morrison at the Human Genetics Unit, MRC, Edinburgh.

##### **2.13.4.1 Probe preparation**

500ng of normal male genomic DNA labelled with digoxigenin was mixed with 500ng of biotin-labelled test DNA and 10µg human Cot-1 DNA. The mix was precipitated in 100µl ice cold ethanol at -70°C for 1 hour and then dried by spinning at 1000 x g in a vacuum centrifuge. At this point, slide preparation (see below) was begun. The DNA pellet was resuspended in 7.5µl CGH buffer, vortexed and left at room temperature for one hour. 7.5µl deionised formamide was added, mixed, pulse-centrifuged and the DNA denatured for 5 minutes at 70°C. Following denaturation, DNA was allowed to reanneal for between 30 minutes and 2 hours at 37°C before being added to the prepared slide.

CGH buffer:

20% dextran sulphate (diluted from autoclaved 50% stock)

4xSSC see 2.12.1.1

##### **2.13.4.2 Slide preparation**

Hybridisation of labelled genomic DNA prepared as above (2.13.4) was carried out on normal male metaphase chromosome preparations which had been stored desiccated for 1-2 weeks (section 2.13.1.2). Treatment was carried out either in 50ml-capacity Coplin jars, moving slides using forceps, or 200ml-capacity glass staining dishes using a slide rack, according to the number to be processed. Use of smaller numbers of slides and Coplin jars was generally found to produce more satisfactory results.

Slides were treated by placing first in RNase A solution at 37°C for 1 hour, washing in 2xSSC at room temperature for 2 minutes and dehydrating through 70%, 90% and 100% ethanol for 2 minutes each. Slides were then warmed on a hotplate to 70°C and placed into 70% formamide solution at 70°C in a fume cabinet for 3 minutes, using a thermometer to keep a constant check on the formamide temperature. This was always carried out in Coplin jars, adding no more than 4 slides at a time to each jar, as this was found to be the best way to maintain a constant solution temperature. Slides were then plunged immediately into ice-cold 70% ethanol and left for 5 minutes before dehydrating through the ethanol series as before.

Finally, they were placed into proteinase K solution at 37°C for 2.5 minutes, washed for 2 minutes in proteinase K buffer at room temperature and dehydrated through ethanol as before.

Prior to adding the probe, slides were placed on a hotplate at 37°C along with small (20mm<sup>2</sup>) coverslips which had been thoroughly cleaned with ethanol. Each DNA mix was placed onto a cover slip, taking care to ensure that the temperature did not drop below 37°C, and the slide placed chromosome-side down on top. The cover slip was sealed with rubber solution and slides were place in a humidified chamber at 37°C for 2-3 days.

RNase A solution:	Proteinase K buffer:	Proteinase K	formamide solution:
10µg/ml RNase A in	0.02M Tris pH 7.5	solution:	35ml deionised
2xSSC	0.002M calcium	100ng/ml proteinase	formamide
	chloride	K in proteinase K	2xSSC
		buffer	
2xSSC:	Deionised formamide:		
see 2.12.1.1	500ml formamide added to 25g ion-exchange resin beads, stirred for 45 minutes, then filtered through a paper filter. Stored at -20°C.		

#### 2.13.4.3 Antibody detection of labelled probes

After incubation, slides were removed from the chamber the rubber sealant carefully removed. They were washed four times for 3 minutes in 50% formamide/2xSSC at 45°C, allowing the cover slips to float off in the first wash. This was followed by four 3 minutes washes in 2xSSC at 45°C and then four 3 minutes washes in 0.1xSSC at 45°C. The slides were then washed for 2 minutes in 0.1% Tween 20/4xSSC at room temperature prior to the addition of antibody.

100µl blocking buffer was added to each slide and covered with a clean large cover slip. After 5 minutes this was drained and 100µl fluorescein/rhodamine solution added in the same manner. Slides were incubated for 30 minutes in the dark at 37°C in a damp chamber. They were then drained and washed three times for 2 minutes in 0.1% Tween 20/4xSSC at 37°C. The slides were drained and 100µl biotinylated anti-avidin (BAA) secondary antibody added and incubated under a cover slip for 30 minutes as before. Slides were washed again three times in 0.1% Tween 20/4xSSC, drained and 100µl fluorescein solution added for a further 30 minutes as before. The slides were then washed three times in 0.1% Tween 20/4xSSC as before, drained and allowed to dry. They were mounted in 35µl DAPI/antifade solution under a large coverslip, sealed with nail varnish, and left overnight in the dark at 4°C before analysis.

Blocking buffer:	Fluorescein/rhodamine solution:
0.5g skimmed milk	1ml blocking buffer
2ml 20xSSC	5µg/ml fluorescein-avidin DCS
made to 10ml with DDW and heated to 50°C to dissolved, aliquoted into 1.5ml tubes and spun at 10000 x g for 15 minutes at 4°C.	Spun at 10000 x g for 15 minutes at 4°C prior to addition of 10µg/ml anti-digoxygenin rhodamine
BAA solution:	Fluorescein solution:
0.5ml blocking buffer	1ml blocking buffer
4µg/ml biotinylated anti-avidin	5µg/ml fluorescein-avidin DCS
Spun 10000 x g for 15 minutes at 4°C	Spun at 10000 x g for 15 minutes at 4°C
DAPI/antifade solution:	
0.75µg/ml DAPI in Vectashield antifade solution	

### 2.13.5 Analysis of CGH

Hybridisations were analysed using the Apple MacIntosh-based quantitative image analysis software MacProbe (Perceptive Scientific Instruments, Ltd). This system uses a standard Leica fluorescence microscope equipped with a charged coupled device (CCD) camera, P-1 filter set and computer-controlled filter wheel for image acquisition. Each fluorochrome is excited separately using a single band-pass excitation filter and gray-level images are captured through a triple bandpass emission filter at  $\times 1000$  magnification, overlaid and displayed in pseudo-colour. A single colour DAPI image is used for chromosome identification. Between five and ten metaphase spreads were analysed for each tumour. Green/red ratios were generated for all undamaged and non-overlapping chromosomes and an average profile was generated for each chromosome using a minimum of 5 autosomes and 3 sex chromosomes. Green/red ratio cut-off points of 1.125 and 0.875 were chosen for scoring of chromosome copy number changes. This was the level at which copy number changes could easily be visualised, and is equivalent to a loss or gain of one chromosome in 25% of cells in a diploid karyotype. Batches were only used if the standard deviation of the sample and the normal control were small. Telomeres, pericentric regions and heterochromatic regions sometimes fell out of range because of the blocking effect of the Cot-1 DNA, and were excluded from analysis.

Characteristics of the fluorochromes used are shown in Table 8.

**Table 8 Characteristics of fluorochromes used in CGH**

Fluorochrome	Optimal excitation $\lambda$ (nm)	Optimal emission $\lambda$ (nm)	Colour
4'-6-diamidino-2-phenylindole (DAPI)	355	450	Blue
Fluorescein	490	520	Green
Rhodamine	545	575	Red

## 2.14 Analysis of tumours from knockout mice

Tumours from mice deficient in *Msh2*, *p53*, or both, were assessed for chromosomal abnormalities by CGH (see Chapter 6). In addition, the status of a number of these tumours was assessed for microsatellite instability. These methods are described below.

### 2.14.1 Generation of nullizygous mice

A large cohort of *Msh2*  $+/+$ , *Msh2*  $+/-$  and *Msh2*  $-/-$  animals were generated on an outbred background segregating for a variety of genomes including 129/Ola, SWR and C57BL/6J. These mice were bred from a single *Msh2*  $+/-$  male, in which removal of *Msh2* activity was achieved by insertion of an intragenic *neo* cassette (de Wind *et al.*, 1995). *Msh2*  $-/-$  mice were interbred with *p53*  $-/-$  mice (Clarke *et al.*, 1993) to generate animals mutant for both genes. Mice were maintained under non-barrier conditions and given a standard diet. Genotype was confirmed by Dr N.Toft and Mr O.Sansom using PCR-based assays specific for targeted and wild-type alleles for both *p53* (Malcomson *et al.*, 1997) and *Msh2* (Dr N.Toft, unpublished). On tumour development (see Chapter 6, Results), mice were killed, the tumour removed and DNA extracted as in 2.3.1.

Generation of mice was carried out under the direction of Dr Alan Clarke.

### 2.14.2 Comparative Genomic Hybridisation

#### 2.14.2.1 Cell lines and metaphase preparation

Normal metaphases for CGH were obtained from the murine embryonic stem cell line E14, derived from mouse strain 129/Ola (Hooper *et al.*, 1987). Cells were grown in CM5-5 medium in 75cm<sup>2</sup> flasks. For metaphase preparation, culture medium was changed 24 hours before cells were 50% confluent (in log-phase growth) to achieve a degree of synchronisation. One hour before harvesting, colcemid was added to the culture medium to a final concentration of 0.1µg/ml. After this time, the medium was removed and kept, and the cells

rinsed twice with PBS. Sufficient TVP was added to cover the bottom surface of the flask, and when cells had disaggregated (about 5 minutes), they were dislodged from the flask by tapping. The spent medium was then added to the cells again to neutralise the trypsin, and the cell suspension spun at  $1000 \times g$  for 5 minutes in a 50ml polypropylene tube. The cells were then gently tapped to resuspend, 50ml 0.075M KCl added and incubated for 10 minutes at room temperature. Cells were centrifuged as before, resuspended in a single drop of supernatant by tapping, and fixed by the dropwise addition of 50ml of ice-cold fix (2 parts methanol to one part glacial acetic acid). The cell suspension was incubated on ice for 30 minutes, centrifuged and resuspended in 50ml of fresh fix. After a five minute incubation, the cell suspension was spun again and resuspended in a small volume of fix to make a milky solution. Slides were prepared and stored as in 2.13.1.2.

TVP:

0.025% (w/v) trypsin

1mM EDTA

1% chick serum (Life Technologies) in PBS.

Sterilised by filtration through 0.2 $\mu$ m filter.

Stored at -20°C.

PBS:

see 2.6

#### **2.14.2.2 Chromosome counts.**

In order to check chromosome number, slides were stained for 30 minutes in 5% Giemsa prepared in PBS. Twenty metaphase spreads were selected at random and the number of chromosomes counted in each spread.

PBS:

see 2.6

#### **2.14.2.3 Nick translation of murine DNA**

Nick translation reactions to label test DNA with biotin were carried out as in 2.13.3. Similarly, an equal amount of DNA from normal mouse tail tissue, extracted as in 2.3.1., was labelled with digoxigenin. Reaction product sizes were checked on an agarose gel as in 2.4.

#### **2.14.2.4 Hybridisation and antibody detection of labelled DNA**

500ng mouse tumour DNA labelled with biotin was mixed with 500ng normal mouse DNA labelled with digoxigenin and 20 $\mu$ g mouse Cot-1 DNA, precipitated, and prepared for hybridisation as in 2.13.4.1. Meanwhile, slides were denatured, dehydrated, and treated with proteinase K and RNase prior to hybridisation with labelled DNA as in 2.13.4.1. Antibody detection of labelled DNA and DAPI counterstaining was as described in 2.13.4.3.

#### **2.14.2.5 Analysis of CGH**

Hybridisations were analysed using the Apple MacIntosh-based Quantitative Image Processing System (QUIPS) software (Vysis, UK, Ltd). The system is based on a normal fluorescent microscope (Zeiss Axioskop) equipped with a CCD camera (Sensys) and triple bandpass filter set for rhodamine, fluorescein and DAPI very similar to the system described in 2.13.5. Because mouse karyotyping software was not available, karyotyping was carried out manually onto a human karyotype template (using only chromosome templates 1-19, X and Y), and thereafter proceeding as in 2.12.5 for generation of green:red ratio profiles. Between four and eight metaphase spreads were analysed for each tumour. Green/red ratio cut-off points of 1.2 and 0.8 were chosen for scoring of chromosome copy number changes. Telomeres and centromeres were excluded from analysis.

#### **2.14.3 Microsatellite analysis of mouse tumours**

In order to assess the status of microsatellite instability in selected tumours compared with paired normal tail tissue, four highly polymorphic microsatellite loci, D1Mit4, D7Mit17, D10Mit2 and D14Mit15 were examined. Primers sequences for these loci were obtained from the Whitehead Institute for Biomedical Research/Massachusetts Institute of Technology Centre for Genome Research website at <http://www.genome.wi.mit.edu> and are shown in Table 9. Reactions were carried out in a 96-well plate and conditions consisted of a final concentration of 1x buffer, 1.5mM MgCl<sub>2</sub>, 200μM each dNTP, 0.5μM each primer, 0.625 units *Taq* DNA polymerase and 25ng DNA template. Reaction volume was made up to 25μl with distilled deionised water (DDW) and one drop of paraffin oil was overlaid to prevent evaporation. After an initial 2 minute denaturation at 95°C, 30 cycles of amplification were carried out with each cycle consisting of 30 seconds denaturation at 95°C, 30 seconds annealing at 57°C and 30 seconds extension at 72°C, followed by final extension at 72°C for 9 minutes. Microsatellite instability was assessed on a 6% denaturing polyacrylamide gel with silver staining as in 2.7.2.

**Table 9**  
**Primer sequences for amplification of mouse microsatellite loci**

Locus	Approximate product size (bp)	Primer sequences (5'-3')
D1Mit4	193	GCTACTGCTTTGGAGTCAGT ATGACTTGAGCTCAGTCTCTG
D7Mit17	161	CTGGCATTATGTTGCTTCA AACTTGCCTTCTGTCCTCCA
D10Mit2	123	CTGCTCACAACCCATTCCTT GTTCAATTTGAGGCACAAGCA
D14Mit15	151	TTGGCTGCTCACTTGCAG TTACCCTCCCCATAACTCCC



## CHAPTER 3

### MICROSATELLITE INSTABILITY AND THE ROLE OF HMSH2 IN SPORADIC COLORECTAL CANCER.

#### 3.1. Introduction

Tumours from hereditary non-polyposis colorectal cancer (HNPCC) patients are characterised by the presence of multiple replication errors (Ionov *et al.*, 1993; Aaltonen *et al.*, 1993). As a consequence of defects in the mismatch repair pathway in such tumours, mutations remain unrepaired. Within microsatellite sequences such replication slippage is manifest as an alteration in length, termed microsatellite instability (MSI), and tumours are termed replication error positive (RER+) when instability occurs at more than one locus (Aaltonen *et al.*, 1993). MSI has also been demonstrated in sporadic colorectal cancers (Thibodeau *et al.*, 1993) and subsequently in many other cancers, including pancreatic (Han *et al.*, 1993), gastric (Han *et al.*, 1993; Peltomaki *et al.*, 1993; Mironov *et al.*, 1994; Rhyu *et al.*, 1994; Chong *et al.*, 1994), prostatic (Gao *et al.*, 1994), endometrial (Risinger *et al.*, 1993; Peltomaki *et al.*, 1993), breast (Patel *et al.*, 1994), non-small cell (Shridhar *et al.*, 1994; Fong *et al.*, 1995) and small cell lung cancers (Merlo *et al.*, 1994) and in Muir-Torre syndrome (Honchel *et al.*, 1993).

Up to 86% of tumours from HNPCC patients exhibit instability at multiple microsatellite sites (Aaltonen *et al.*, 1993 & 1994; Wu *et al.*, 1994). The proportion of sporadic colorectal cancers which demonstrate the same phenomenon, though much lower (around 17% in most studies), nevertheless accounts for a significant number of cancer patients (Aaltonen *et al.*, 1993 & 1994; Ionov *et al.*, 1993; Lothe *et al.*, 1993; Young *et al.*, 1993b; Thibodeau *et al.*, 1993; Kim *et al.*, 1994a). Sporadic tumours with microsatellite instability have characteristics in common with HNPCC tumours. They are usually located in the proximal colon (Ionov *et al.*, 1993; Thibodeau *et al.*, 1993; Lothe *et al.*, 1993; Aaltonen *et al.*, 1993; Kim *et al.*, 1994a), associated with extracellular mucin production (Kim *et al.*, 1994a), poor differentiation (Ionov *et al.*, 1993; Lothe *et al.*, 1993; Kim *et al.*, 1994a) and near-diploid DNA content (Aaltonen *et al.*, 1993; Lothe *et al.*, 1993; Remvikos *et al.*, 1995; Schlegel *et al.*, 1995). They also show a negative correlation with mutation (Ionov *et al.*, 1993) or immunochemical stabilisation of p53 (Kim *et al.*, 1994a) and Ki-ras mutation (Ionov *et al.*, 1993). In addition, loss of heterozygosity at known tumour suppressor gene loci on chromosomes 5q, 17p and 18q is relatively less frequent in tumours with MSI compared to those without (Thibodeau *et al.*, 1993). Finally, these tumours show a tendency towards increased patient survival (Thibodeau *et al.*, 1993; Lothe *et al.*, 1993).

Recently, a number of genes that participate in human mismatch repair have been identified, namely hMSH2 (Fishel *et al.*, 1993; Leach *et al.*, 1993), hMLH1 (Bronner *et al.*, 1994; Papadopoulos *et al.*, 1994), hPMS1 and hPMS2 (Nicolaidis *et al.*, 1994), hMSH6 [also called p160 or GTBP] (Palombo *et al.*, 1995; Drummond *et al.*, 1995; Papadopoulos *et al.*, 1995) and hMSH3 (Risinger *et al.*, 1996). The roles of these genes were inferred from their homology with bacterial and yeast mismatch repair genes (Prolla *et al.*, 1994), and germline mutations have been found in HNPCC families. The most frequently involved are hMSH2, where mutations account for at least 50% of HNPCC kindreds (Peltomaki *et al.*, 1993; Liu *et al.*, 1994; Nystrom-Lahti *et al.*, 1994; Wijnen *et al.*, 1995; Liu *et al.*, 1996a), and hMLH1, which accounts for up to 40% (Han *et al.*, 1996; Liu *et al.*, 1996a). Mutations in these genes may also play a role in the development of sporadic colorectal cancer. However, mutations have only been demonstrated in a small percentage of sporadic cancers or cell lines derived from colorectal carcinomas so far (Liu *et al.*, 1995b; Borresen *et al.*, 1995; Bubb *et al.*, 1996; Moslein *et al.*, 1996; Herfarth *et al.*, 1997).

The aim of this study was to clarify the prevalence of mismatch repair deficiency in a large unselected series of 215 Scottish sporadic colorectal cancer cases and relate this to clinico-pathological factors. Secondly, to delineate the role of a specific mismatch repair gene known to be important in hereditary cancers, extensive mutation analysis of hMSH2 was carried out on selected cases. In the course of this, mutations were identified not only in hMSH2 exons, but also in adjacent intronic sequence. A further series of experiments was carried out to establish whether these intronic mutations were likely to be purely secondary to mismatch repair (engendered by defects in some other gene) or might affect the function of hMSH2, perhaps through altered splicing.

## 3.2. Materials and Methods

### 3.2.1. Outline of methods

#### 3.2.1.1 Analysis of microsatellite instability

The initial part of this study examined microsatellite instability in a large series of 215 sporadic colorectal carcinoma patients, comparing tumour with matching normal tissue. Each tumour was examined for instability in at least four microsatellite loci, using a combination of automated laser fluorescence (ALF), silver staining and radioactive isotope labelling. Detailed methods can be found in 2.7. Clinical and pathological features of these tumours (Dukes' stage, tumour site, p53 status by immunocytochemistry [see 2.6], histological features) were assessed and their incidence in cancers with unstable microsatellites versus those with stable microsatellites tested. Patient survival in these two groups was also compared using the Cox proportional hazard method.

#### 3.2.1.2 Statistical analysis

The effect on post-operative survival of all relevant factors was investigated by analysis with the Cox proportional hazards method, using the Cox Regression routines of SPSS for Windows 6.0. Post-operative survival was established by perusal of death certificates held by the Registrar General for Scotland. Those patients who did not appear in the registry on the census date were traced through hospital records to confirm survival by continuing out-patient attendance or by letter to their family practitioner. The censoring date for survival was taken as 31.12.93; thus, all who survived later than this date were treated as 'last known alive' at this time. Factors investigated as possible predictors of survival were age, tumour side, p53 status, gender, shift status and Dukes stage. Deaths from all causes were considered.

#### 3.2.1.3 Mutation analysis of hMSH2

Details of methods are presented in 2.8 and 2.9. Following identification of 39 cancers with microsatellite instability, 36 were taken for mutation analysis of hMSH2, which was carried out using a combination of single strand conformation polymorphism (SSCP) analysis and heteroduplex analysis. Exonic and, where possible, splice site sequences were analysed by one or both of these methods. A restriction digest assay was used to detect a previously reported polymorphism in hMSH2 intron 12 (Fishel *et al.*, 1993; Hall *et al.*, 1994) and a new polymorphism in exon 1. All tumours showing aberrantly migrating bands by SSCP or heteroduplex analysis and their corresponding normal DNA were sequenced using the dideoxy chain termination method, either directly from PCR product, from a PCR product made single-stranded by use of a biotinylated primer and streptavidin-coated magnetic beads or after cloning into a vector (see 2.9). In cases where single *Taq* polymerase-amplified

clones were analysed, further sequencing was carried out using high-fidelity polymerase as the rate of *Taq* polymerase-induced errors was found to be high.

Two intronic regions of the hMSH2 gene were found to harbour frequent mutations in repetitive sequences, postulated to affect mRNA by causing aberrant splicing or reducing the levels of transcript. Although frozen tissue was not available from these tumours for RNA analysis, this possibility was investigated by extraction of RNA from xenografted tumours derived from primary lesions known to contain an intronic mutation, as well as wild-type controls. cDNA was produced by reverse transcription and two assays were carried out. In the first procedure, PCR primers were designed to amplify three regions spanning the mutations to search for evidence of aberrant splicing. Secondly, since insufficient tumour material was available for extraction of RNA in adequate quantities for Northern blotting, a quantitative PCR-based assay was used to ascertain the level of transcript present in mutated versus normal samples. Methods are detailed in 2.10.

### 3.3. Results.

#### 3.3.1. Microsatellite Instability Analysis

##### 3.3.1.1 Characteristics of tumours with microsatellite instability

A total of 215 patients diagnosed as having primary sporadic colorectal cancer were included in this study. This group was unselected, and included patients with more than one tumour and those with cancer at other sites (either synchronous or asynchronous) who did not fit the criteria for HNPCC (Percesepe *et al.*, 1994). When patients had more than one carcinoma, each lesion was analysed separately if DNA was available. Thus, a total of 219 carcinomas were analysed, four patients each having 2 synchronous cancers. Relevant clinical, pathological and genetic details for these patients, as well as their study number for cross-referencing, are given in Appendix 5.

These 219 cancers were staged as follows: 25 Duke's Stage A, 100 Duke's Stage B and 94 Duke's Stage CD (for these purposes stages C and D were amalgamated as information regarding distant metastasis was not always available). The average age was 70 years, range 28-95 years, with one patient of unknown age. The group comprised 46.5% (100/215) males and 53.5% (115/215) females.

All samples were analysed for genetic instability at a minimum of 4 microsatellite loci. 16.4% (36/219) exhibited instability at one or more loci. The average age of this group was 68.75 years, range 37-93 years. This is not significantly different from the age of the group without evidence of MSI (70.02 years, range 28-95). During the course of this study a polyadenine tract located in the 5' region of hMSH2 intron 5 was identified (Figures 5 and 6) which exhibited replication errors in 58% (19/33) of samples showing microsatellite instability at other loci. When instability at this locus was included, a further 3 patients were found whose tumours showed evidence of MSI, one of which appeared to show instability at another locus but despite repetition had not given an unequivocal result. Thus, in total, 17.8% (39/219) of sporadic colorectal cancers in this series exhibited MSI. When the more stringent requirement of instability at 2 or more loci (including intron 5 of hMSH2) was applied, a criterion often used to define a replication error positive (RER+) phenotype (Aaltonen *et al.*, 1993), the incidence of MSI was 10.5% (23/219).

##### 3.3.1.2 Microsatellite instability in relation to clinicopathological features

The relationship between microsatellite instability and site of tumour, p53 stabilisation and Duke's stage was examined (Table 10). 59% (23/39) of lesions with instability at one or more loci were in the proximal or right side of the bowel (ie proximal to and including the splenic flexure). This was significantly different ( $\chi^2 = 7.73$ ,  $p < 0.01$ ) from the distribution of those without MSI (non-shifters), where 35% (63/180) were right-sided, similar to the general distribution of all colorectal cancers in the UK of which 28% are right-

sided (Cancer Research Campaign, 1993). Of lesions with at least 2 unstable sites, 74% were proximal ( $\chi^2=12.93$ ,  $p<0.001$ ). When proximal tumours alone were considered, 27% (23/86) demonstrated microsatellite instability at one or more loci, and 17/86 (20%) were RER+, as compared with only 12% (16/133) of distal cancers.

When evidence of p53 stabilisation was examined in this series of tumours [scored positive if any tumour nucleus in the section stained (Purdie *et al.*, 1991)], 56.4% (22/39) of tumours with MSI at one or more loci stained positively for p53 protein (Figure 4), which was not significantly different from those without MSI, in which 60% (108/180) had evidence of stabilised p53 ( $\chi^2 = 0.43$ ,  $p>0.5$ ). However, a significant inverse relationship was seen between MSI status and p53 staining in tumours with instability at more than one loci ( $\chi^2 = 4.43$ ,  $p<0.05$ ). Fifty-nine percent of the total population had positively stained nuclei. When the site of lesion was considered 54% of proximal tumours and 64.5% of distal tumours had evidence of stabilised p53. These results were compared to data generated from the analysis of a subset of these cases for loss of heterozygosity (LOH) at 17p using three restriction fragment length polymorphic (RFLP) markers intragenic to p53 (Dr C.A.Purdie, personal communication). 125 lesions were analysed for intragenic loss of p53, of which 49 were informative at one or more loci. Of these informative cases, 19 had allele loss involving p53 and in 3 (16%) of these we demonstrated MSI, all located on the left side of the bowel. Of the 30 lesions where heterozygosity in p53 appeared to be retained, 9 (30%) exhibited MSI; of these 6 were located on the right side of the colon. Thus there is an apparent trend for tumours that exhibit MSI to retain heterozygosity in p53, but this difference was not statistically significant. There was no significant difference between Duke's Stage and MSI status in tumours with MSI at one or more locus ( $\chi^2 = 2.37$ ,  $p>0.3$ ) or at more than one loci ( $\chi^2 = 2.028$ ,  $p>0.3$ ) (Table 10), although lesions with MSI appeared to be less common within the Duke's A group.

Histological examination of the 39 tumours with MSI revealed that 41% (16/39) of these tumours contained large lakes of extracellular mucin mixed with tumour cells and could thus be classified as mucinous carcinomas (Rosai, 1996). This is a much higher proportion than in the unselected Scottish population of sporadic colorectal cancers; in such a series overlapping with the present study only 17% (59/346) mucinous carcinomas were observed. Examples of histological tumour types are shown in Figure 3. Degree of differentiation was found to show substantial heterogeneity throughout the tumour, and therefore the sample taken for DNA could not be reliably assessed for this factor.

**Table 10**  
**Characteristics of tumours with MSI.**

MSI STATUS			
	UNSTABLE ≥ 1 LOCUS	UNSTABLE > 1 LOCUS (RER+)	<sup>a</sup> STABLE
<b>SIDE</b>			
LEFT	16 (41%)	6 (26%)	117 (65%)
RIGHT	23 (59%)	17 (74%)	63 (35%)
<b>DUKES STAGE</b>			
A	2 (5%)	1 (4.5%)	23 (13%)
B	21 (54%)	13 (56.5%)	79 (44%)
CD	16 (41%)	9 (39%)	78 (43%)
<b>p53 STATUS</b>			
<sup>b</sup> ICC POSITIVE	22 (56%)	9 (39%)	<sup>d</sup> 108 (60%)
ICC NEGATIVE	17 (44%)	14 (61%)	66 (37%)
<sup>c</sup> UNKNOWN			6
TOTAL	39	23	180

<sup>a</sup>no alteration in length seen at loci tested. <sup>b</sup>immunocytochemistry <sup>c</sup>PLPD fixed tissue unavailable

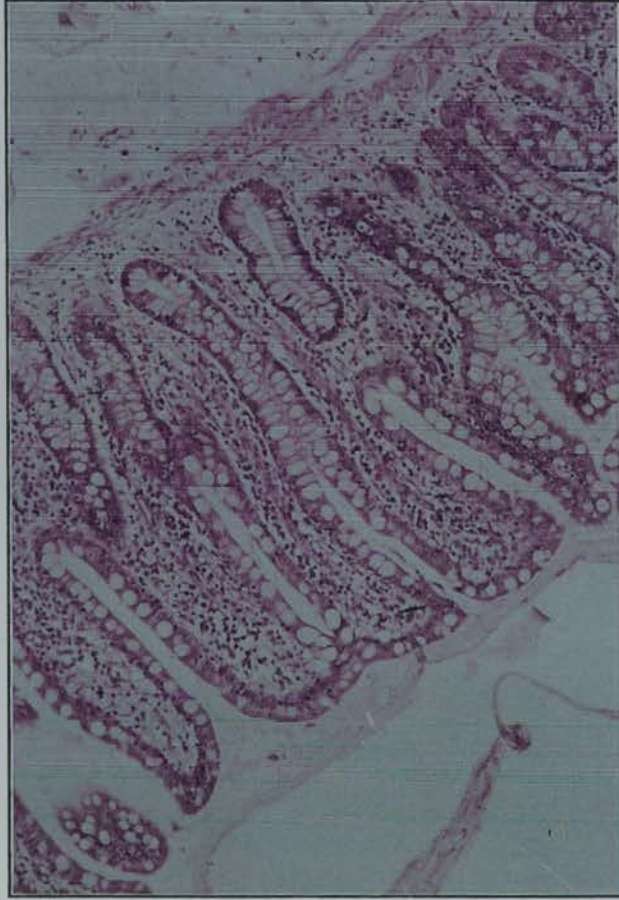
<sup>d</sup>3 samples included with strong cytoplasmic staining, nuclei were not stained.



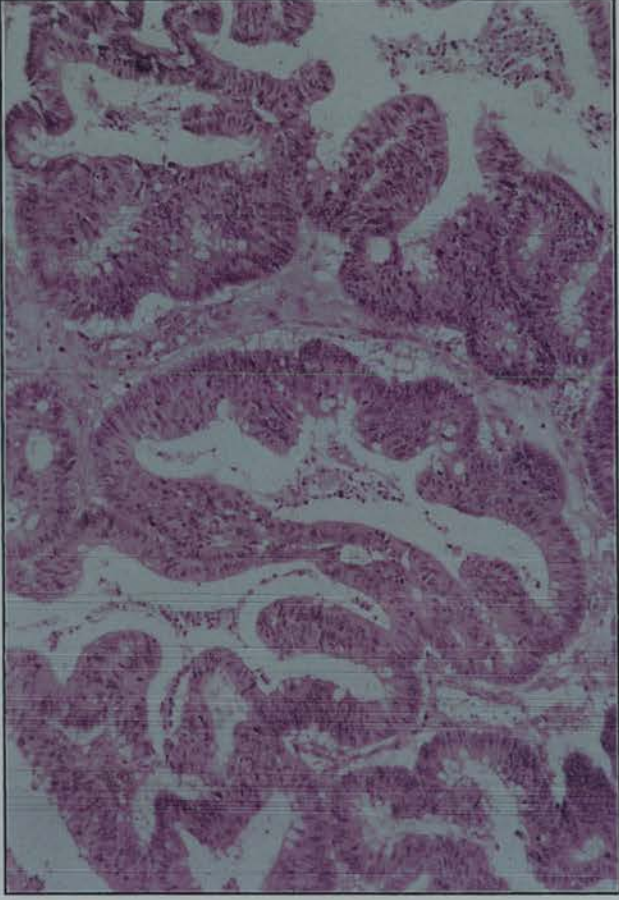
**Figure 3**

**Examples of haemotoxylin and eosin (H&E) staining of sections of paraffin-embedded formalin fixed colonic tissue.**

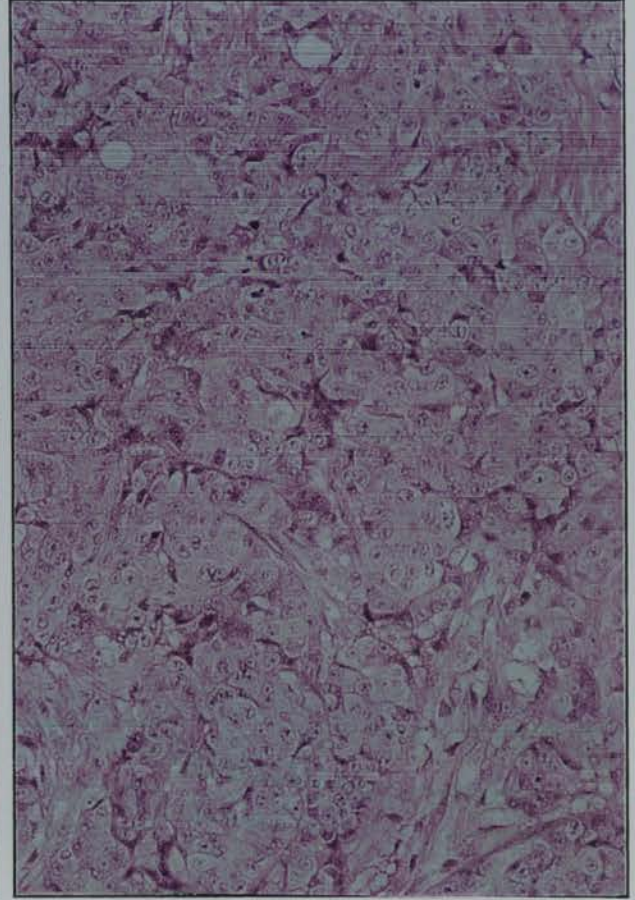
- (A) Normal colonic epithelium.
- (B) Well- to moderately-differentiated adenocarcinoma.
- (C) Poorly-differentiated adenocarcinoma.
- (D) Adenocarcinoma with mucinous histology.



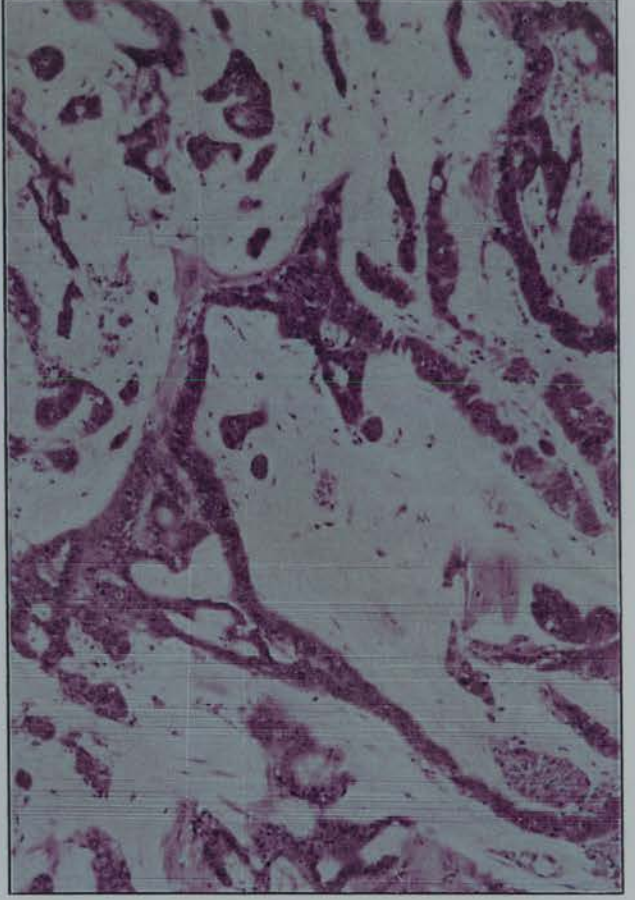
A



B



C



D

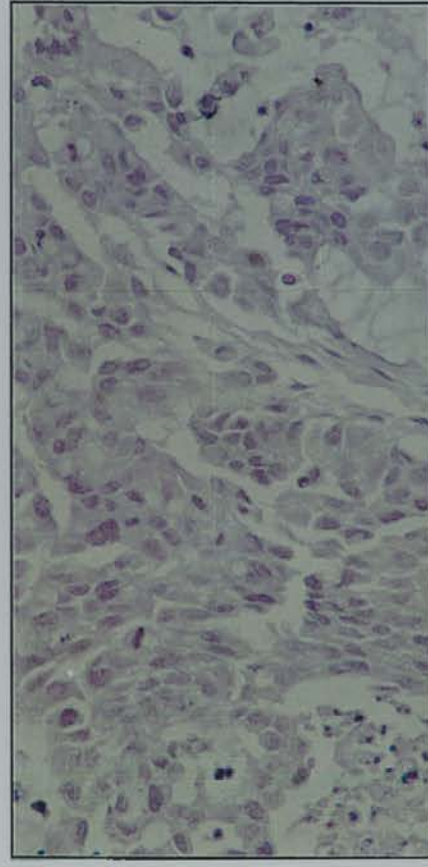
#### **Figure 4**

##### **Immunohistochemical detection of p53 protein.**

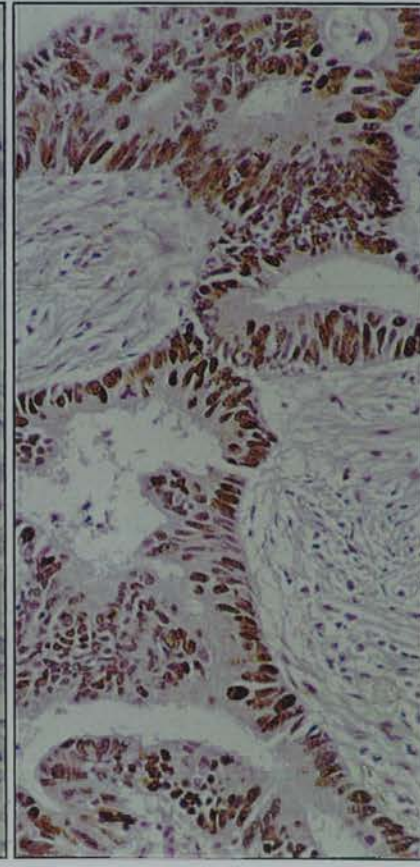
Immunohistochemistry of sporadic colorectal carcinomas using DAB-detection of anti-p53 antibodies DO7 and pAB1801. Bound antibody is stained brown, background cells are stained purple with haematoxylin.

- (A) Tumour negative for the presence of stabilised p53 detected by DO7.
- (B) Strongly positive staining of stabilised p53 by DO7.
- (C) Tumour showing occasional positive staining with DO7. Arrows indicate examples of positive cells.
- (D) Tumour negative for the presence of stabilised p53 detected by pAB1801.
- (E) Strongly positive staining of stabilised p53 by pAB1801.

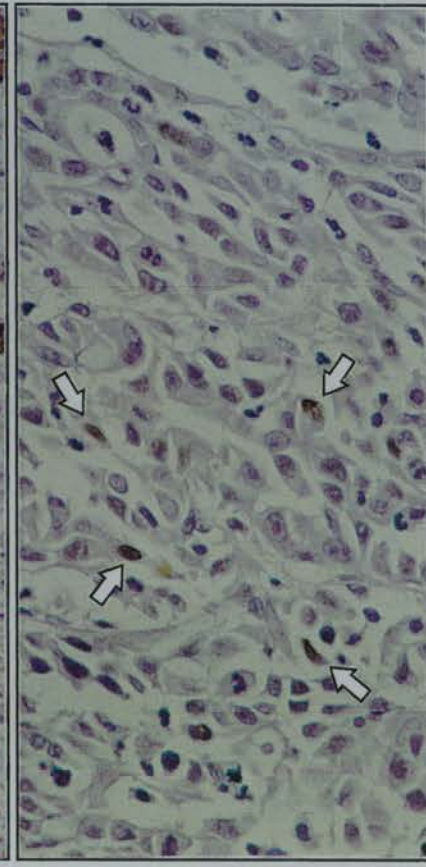




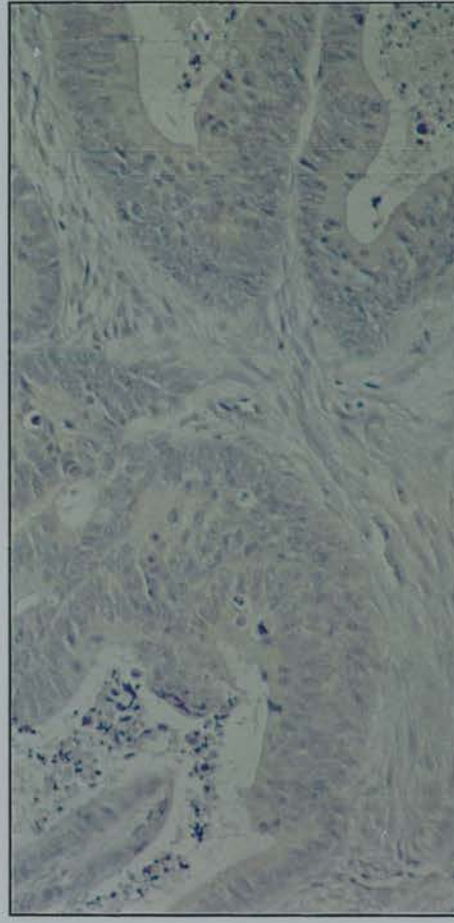
A



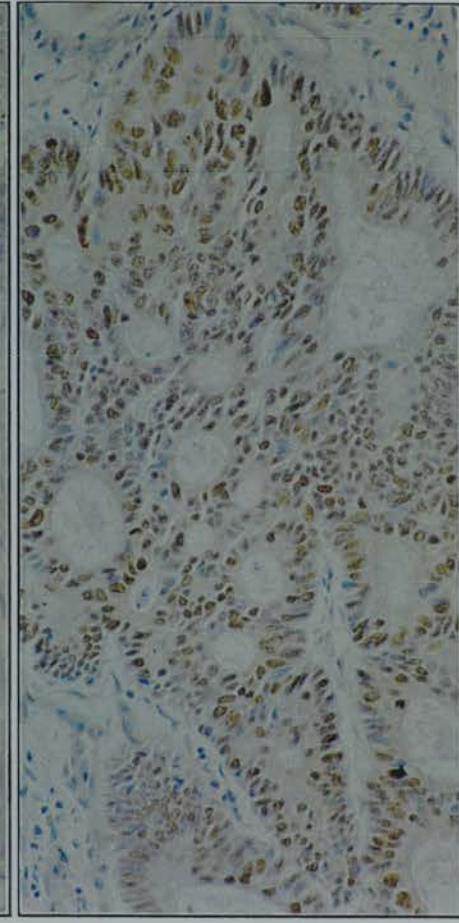
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C



D



E

### 3.3.2. Survival Analysis

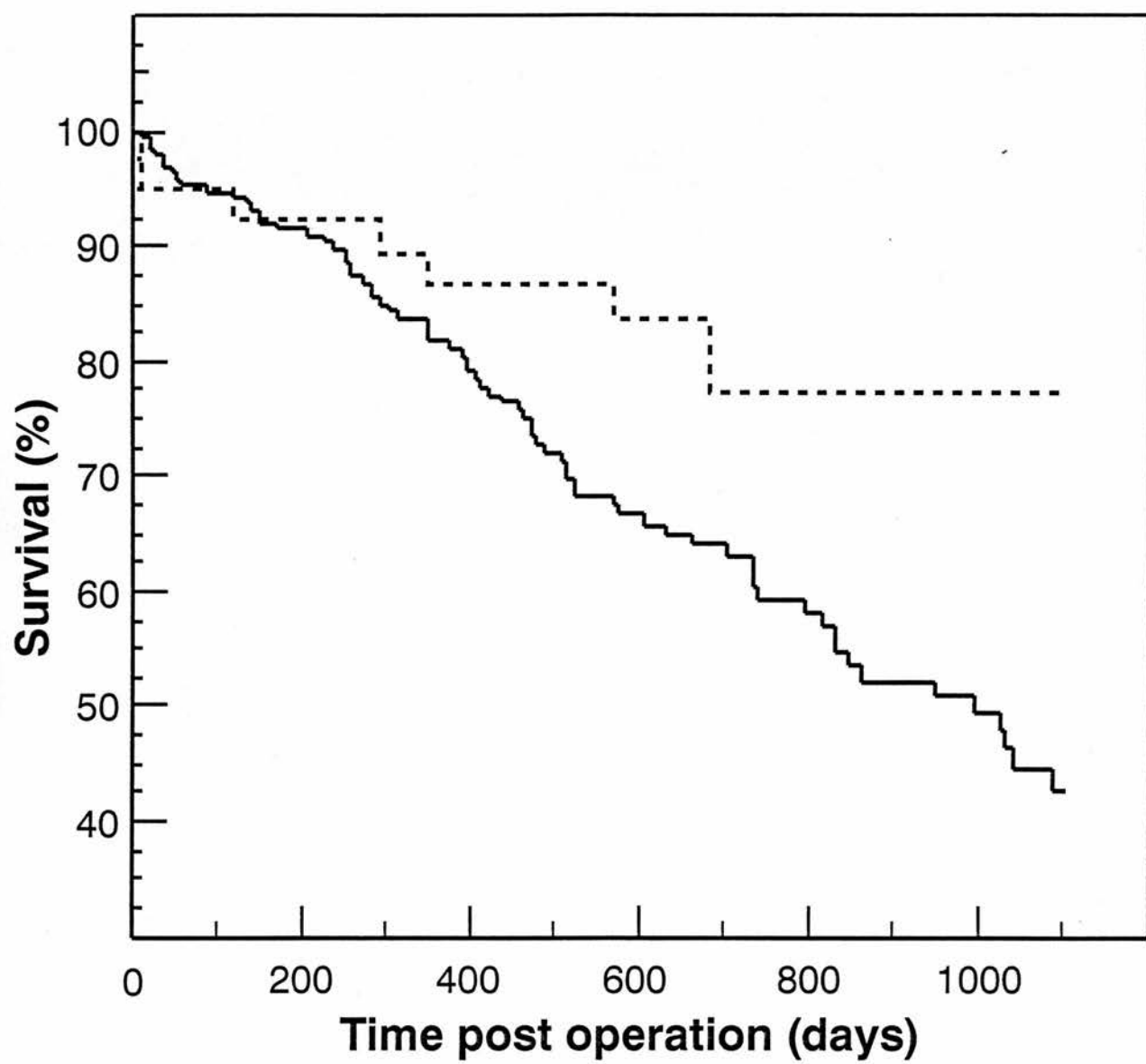
Survival analysis was carried out on 169 patients for whom full information on test criteria (described in 3.2.1.2) was available. Analysis was carried out using the Cox proportional hazards method. Thirty-five of the 169 tumours (20.7%) exhibited replication errors at one or more microsatellite loci. Survival of patients from whom these cancers were resected was markedly above that of patients whose tumours displayed no such instability (77% at 3 years compared with 43%). The most significant predictor of survival was Duke's stage ( $\chi^2=38.4$ ,  $p<0.0001$ ) followed by age ( $\chi^2=9.11$ ,  $p=0.0025$ ), MSI ( $\chi^2=7.83$ ,  $p=0.0051$ ) and side ( $\chi^2=6.35$ ,  $p=0.012$ ). The hazard ratio of patients with tumours showing MSI to those without was estimated to be 0.39 (95% C.I. 0.19-0.82). Thus, at any point in time after diagnosis, a patient whose tumour showed MSI had a risk of dying which was estimated to be about 39% of that of a patient with a tumour without MSI, even after allowing for the influence of other predictive factors. The results are presented as an estimated survival curve (Figure 5). p53 immunohistochemical status and sex showed no association with prognosis.

Cancers arising in the proximal bowel were associated with better prognosis than those arising in the distal bowel, though this effect was less significant than the effects of microsatellite instability. When analysis was repeated excluding 'site of tumour', those with microsatellite instability still had significant survival advantage [hazard ratio of 0.37 (95% C.I. 0.18-0.77)].

**Figure 5**

**Survival analysis of sporadic colorectal cancer patients with and without MSI.**

Estimated survival probabilities for patients with MSI (---) and those without (—) by days post-operation using the Cox proportional hazards method. Probabilities are plotted for a hypothetical set of patients with the same mean effects of Duke's stage, tumour side and age as observed in the study sample. The number of patients with and without MSI surviving at 1100 days were six and 29 respectively.





### 3.3.3. Mutation Analysis of hMSH2

#### **3.3.3.1. Mutations within hMSH2**

A total of 36 tumours with microsatellite instability at one or more loci (including hMSH2 intron 5) were analysed for mutations in the hMSH2 gene using either SSCP mutation analysis, heteroduplex analysis or both methods (Figure 6). In addition, 32 tumours without MSI were analysed as controls. Germ-line exonic mutations were found in only two cases (Table 11 and Figures 7&8). Both of these were mis-sense mutations and both occurred in the control group, one in a 74-year old patient with no family history of disease and one in a 49 year-old with a family history of pancreatic cancer. Both cancers occurred in the proximal colon. Only one tumour with MSI was found to carry any exonic mutation, a frameshift creating a new stop codon. This tumour also showed a reduction in length of a polyadenine tract located 3 nucleotides downstream of the 3' end of exon 5, a change which was found in an additional 21 tumours (Figure 6, 7 & 8 and Table 11). Of these 21 tumours, the polyadenine tract length alteration was found to be somatic in 20 cases but occurred in both the tumour and normal tissue of one patient. These alterations were initially detected by abnormal banding on SSCP gels, but were later found to be easily detectable on denaturing polyacrylamide gels. Abnormal allele lengths ranged from 10 to 23 bases compared with matching normal tissue which had 25 adenine nucleotides in this tract, which differs from the previously published sequence of 26 residues (Liu *et al.*, 1994). This change has been reported by others to occur frequently in colorectal cancers with microsatellite instability (Hoang *et al.*, 1997).

A further tumour with MSI had an intronic transition-type point mutation, an alteration of unknown function, as well as the length reduction in intron 5 and a second reduction in the length of intron 1. Seven other tumours had this somatic reduction in the length of the polythymine tract located 3' in intron 1, predicted to be part of the splice site. Whereas in normal tissue this tract extends for 12 or 13 nucleotides, this was reduced to as few as 9 base pairs in some tumours. Analysis of 6 cancer-free control samples demonstrated that 12 or 13 bases was a normal polymorphic variation (Dr V.J. Bubb, personal communication). Removal of this polynucleotide tract by *Pst* I digestion and repetition of SSCP on the residual 176 nucleotide fragment (containing 90% of the exonic sequence) revealed no further mutations.

When these results were combined, 15 tumours had a single change in the hMSH2 gene, 8 tumours had 2 alterations and one had three. Thus, a total of 61% (22/36) of tumours with MSI had an alteration in hMSH2 compared to 6% (2/32) of non-shifters analysed. However, in only one tumour with MSI was there a hMSH2 mutation within an exon.

### 3.3.3.2. Polymorphisms within *hMSH2*

Two alleles were detected by heteroduplex analysis in intron 1, which sequencing revealed to be a C→G substitution at the +9 position (Figures 6&7). This destroyed a *Bcn* I cleavage site, permitting use of a PCR-RFLP assay to calculate allele frequency in 106 unselected healthy blood donors. The allele frequencies were A1= 0.38 and A2= 0.62 in this local population. The same frequency for each allele was found for the cases analysed for *hMSH2* mutation in our study population. Thus this polymorphism does not appear to influence susceptibility to sporadic colorectal cancer.

Twenty-seven percent of patients with MSI were heterozygous for a previously described C/T polymorphism at the -6 position of intron 12 (Fishel *et al.*, 1993; Hall *et al.*, 1994) [Figure 7]. Two patients carried both the polymorphism described at +12 position of intron 10 (Wijnen *et al.*, 1994) and the polymorphism described at -9 position of intron 9, GTCGTT-GTCATT (Borresen *et al.*, 1995) [Figure 7].

Table 11. Mutations detected within the hMSH2 gene in sporadic colorectal cancers.

PATIENT STUDY NO.	EXON/ INTRON	CODON	MUTATION TYPE	MUTATION GENOTYPE	MSI STATUS
33	1	codon 21 CAC→CAG His-Gln	mis-sense	germ-line	stable
19	2	T→C, -19 position of intron 1 3' end	intronic	somatic	shift
69	3	codon 102 AAT→AGT Asn-Ser	mis-sense	germ-line	stable
51	15	codon 818 deleted T, creates stop codon downstream	nonsense	somatic	shift
9,19,26,28,53,81,85,89	2	reductions in polythymine tract length, 3' end intron 1	intronic	somatic	shift
1,3,17,19,26,28,34,35,50,51,53,61,62,81,82,84,85,86,88,89	5	reductions in polyadenine tract length, 5' end intron 5	intronic	somatic	shift
9				germline	shift

Patient study numbers are given in Appendix 5.

## Figure 6

### Mutation detection methods in hMSH2

Examples of mutation detection of (A) a deletion of 15 adenine bases from the polyadenine tract of

intron 5 and (C) a polymorphic C/G base change within intron 1 (sequence from normal tissue of two individuals, homozygotes for A1 and A2, are shown).

(B) shows **SSCP analysis** of intron 5 for three normal/tumour pairs, where sample 1 is the same pair as shown in A, sample 2 has no mutation and sample 3 (patient no.9 in the study) carries a germ-line change.

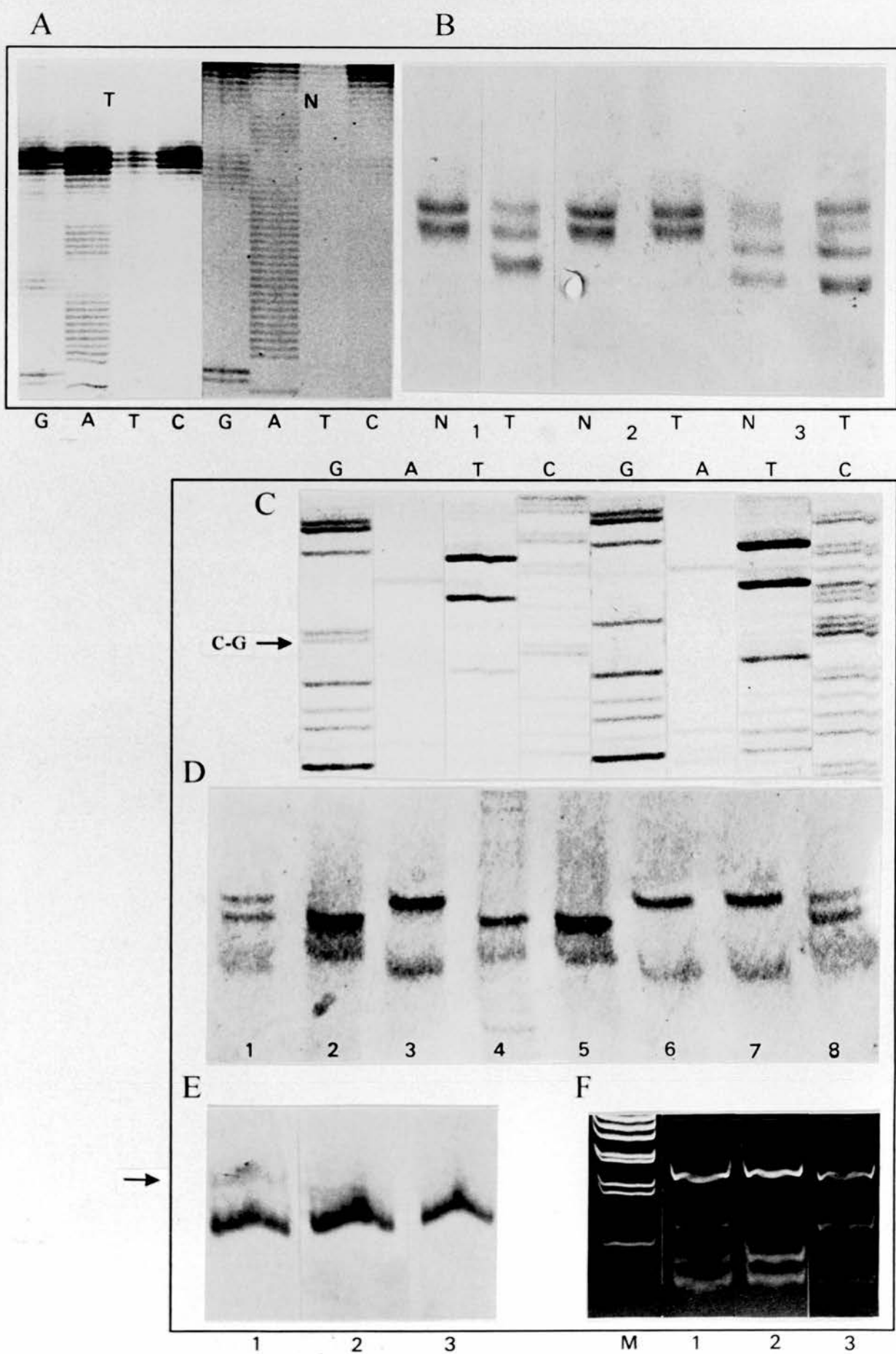
(D) **SSCP analysis** of the polymorphism shown in C, where samples 1 and 8 are heterozygous, samples 2, 4 and 5 are homozygous for A2 and samples 3, 6 and 7 are homozygous for A1.

(E) **Heteroduplex analysis** of the first 3 samples shown in D, with the heteroduplex band marked by an arrow.

(F) The same 3 samples detected by **Bcn1 digest**. Allele sizes for *Bcn1* digests are 81bp (A1) and 57bp (A2). The lower 48bp and upper 152bp bands are constant. A 21bp constant band is not shown.

M=1kb ladder DNA molecular weight marker (Life Technologies Ltd).

T= tumour, N=normal tissue



## Figure 7

### Sequencing of hMSH2 mutations and polymorphisms.

Dideoxy chain termination sequencing of all mutations (A-F) and polymorphisms (G-J) in hMSH2 detected in this study (see table 11 and Figure 8).

(A) Sequencing of a single cloned allele showing a germ-line C-G transversion at codon 21, exon 1, in tumour (right) and normal (left) tissue from patient 33.

(B) T-C transition at the -19 position of the 3' end of intron 1 in the tumour (right) from patient 19, not present in matched normal tissue (left). This patient also has a 4bp reduction in the length of the intron 1 polythymine tract, illustrated in another patient in (E).

(C) Germ-line A-G transition in codon 102, exon 3, in patient 69 (normal tissue on the left, tumour on the right).

(D) Single thymine deletion in codon 818, exon 15, in tumour (right) of patient 15, creating frameshift and stop codon.

(E) Somatic deletion of two thymines in the polythymine tract at the 3' end of intron 1 in patient no. 85 (tumour on right, normal tissue on left).

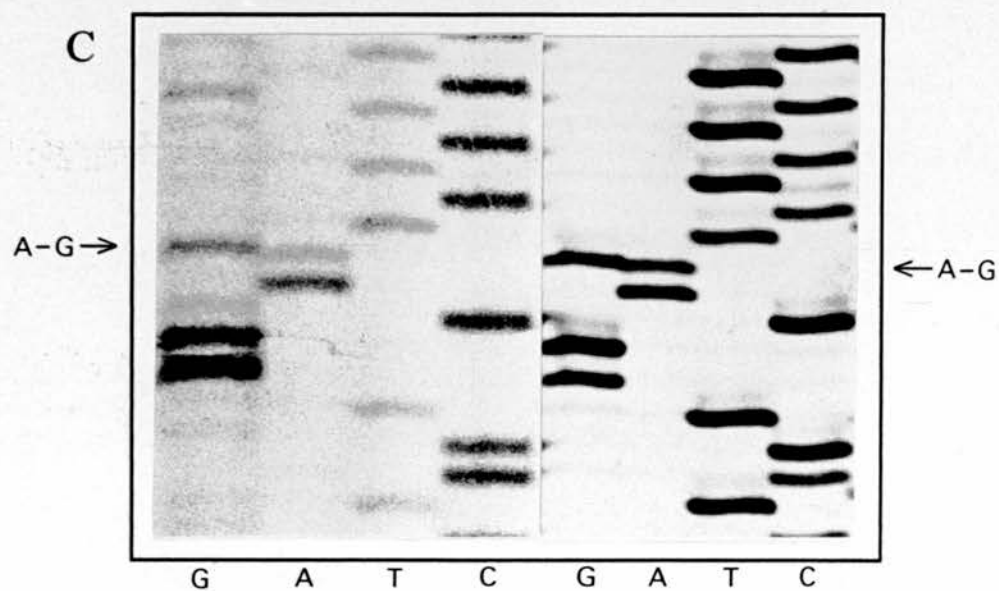
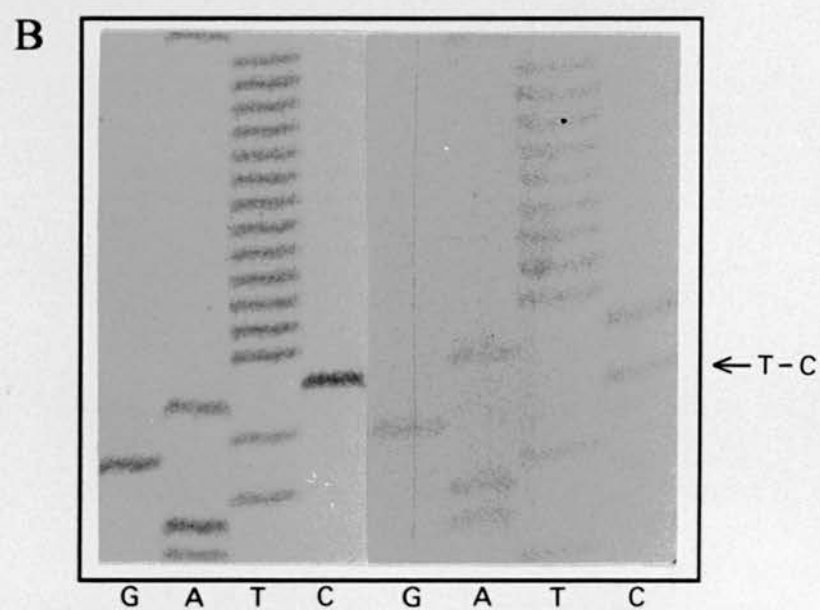
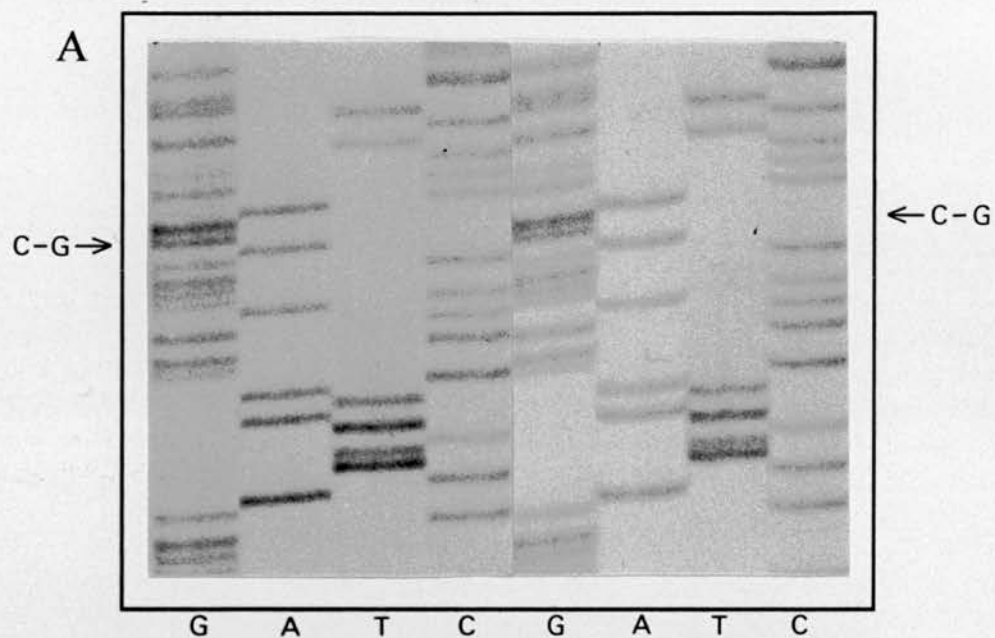
(F) Deletion of 15 adenines in the polyadenine tract at the 5' end of intron 5 in tumour material (right) from patient no. 34.

(G) C/G polymorphism at the +9 position of intron 1 shown in homozygote A1 (patient 38, left) and homozygote A2 (patient 6, right).

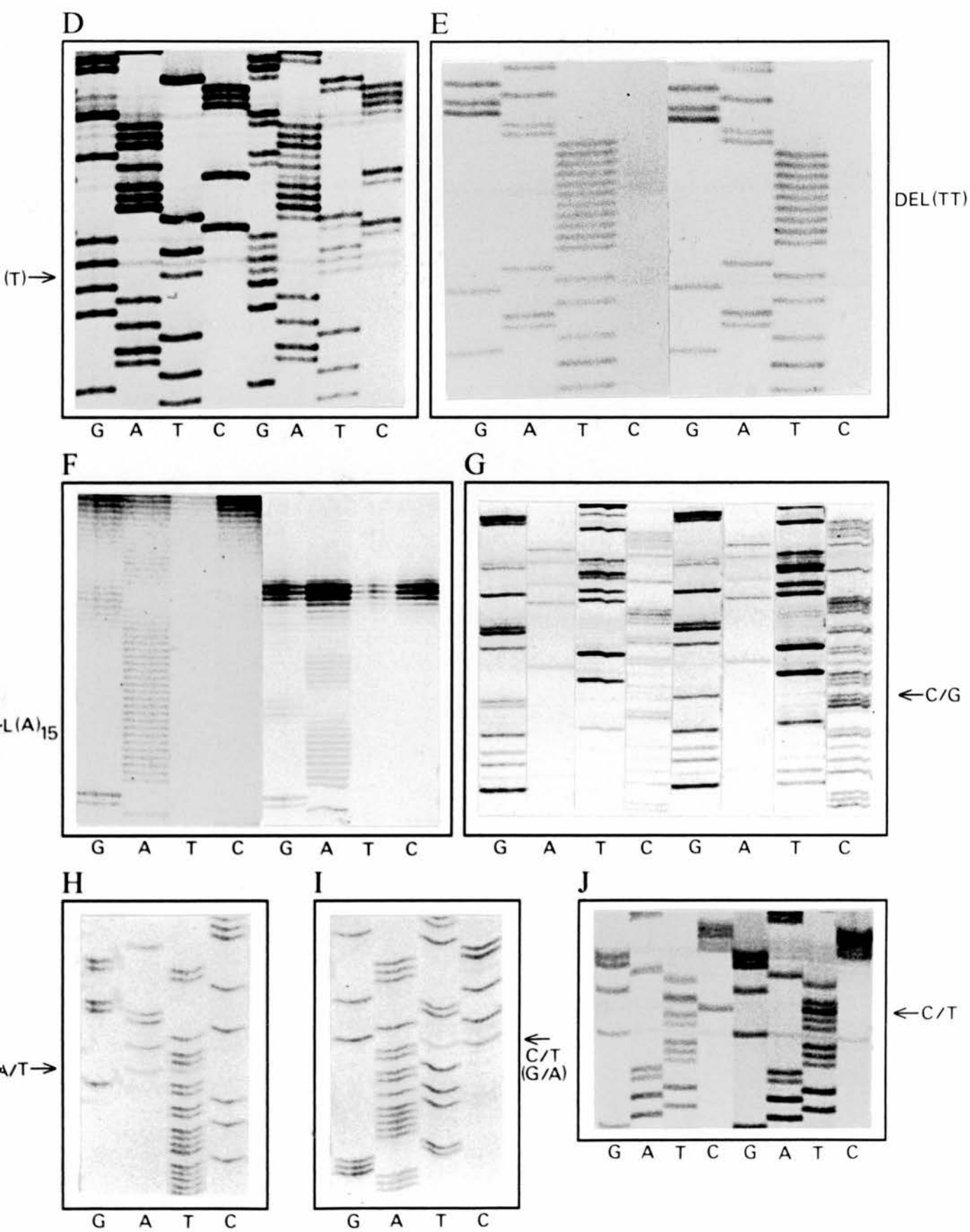
(H) Normal tissue from patient 84, heterozygous for an A/T polymorphism at the +12 position of intron 10 (Wijnen *et al.*, 1994).

(I) Reverse strand sequencing of normal tissue from patient 84, heterozygous for a G/A polymorphism at the -9 position of intron 9 (Børresen *et al.*, 1995).

(J) Normal tissue from patient 81, heterozygous for a C/T polymorphism at position -6 of intron 12 (Fishel *et al.*, 1993; Hall *et al.*, 1994).

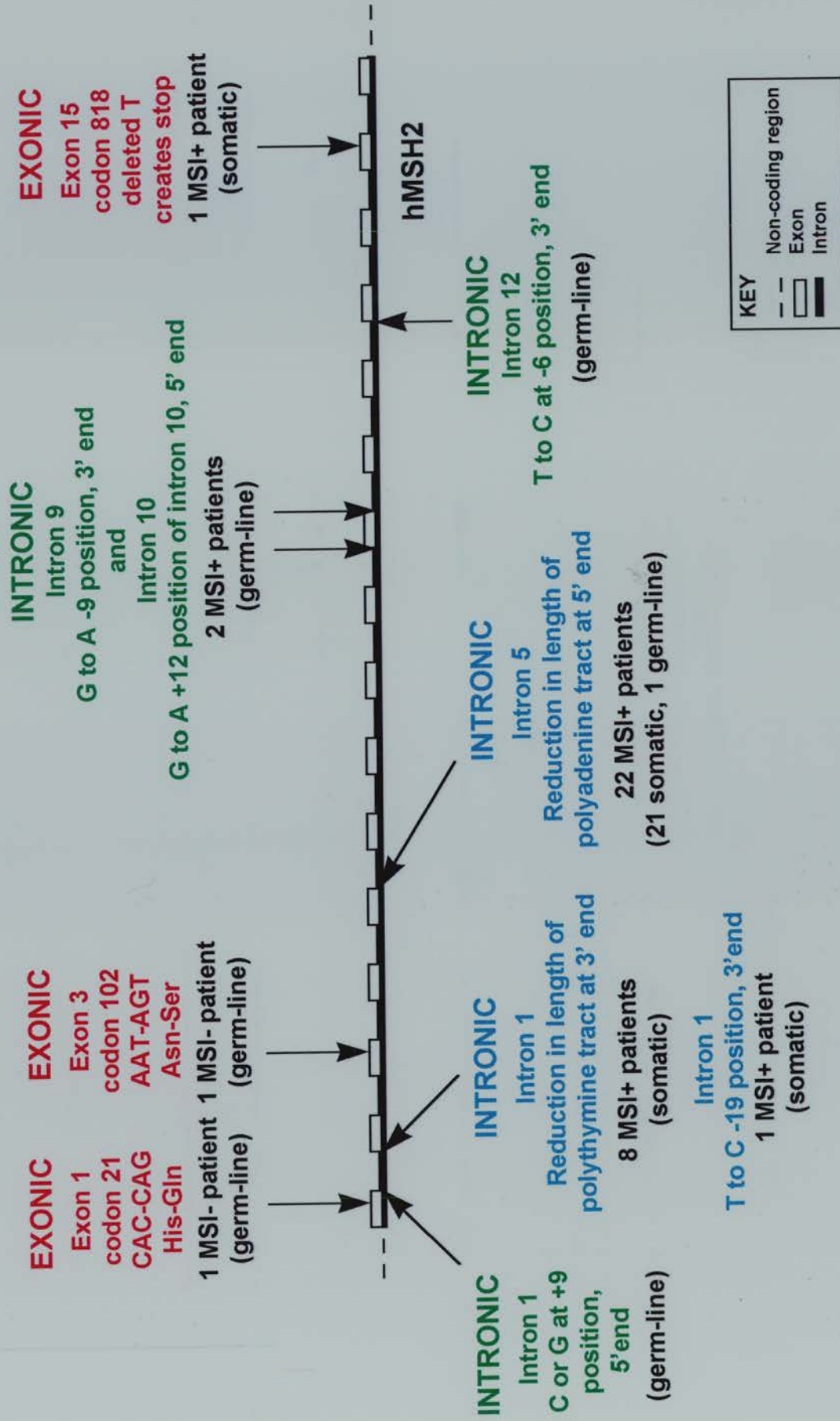






**Figure 8**  
**Location of mutations and polymorphisms within hMSH2**

Schematic representation of Table 11, showing the location of mutations and polymorphisms within in hMSH2.



### 3.3.3.3. Intronic sequence alterations in hMSH2 and transcription

The two most common intronic sequence alterations detected in hMSH2, the polynucleotide tract length changes in introns 5 and 1 (Table 11 and Figures 6,7 & 8), were investigated for their effects on transcription of the hMSH2 gene. Xenografted tumours were used for this analysis, since RNA was not available from primary tumour tissue. RNA from at least 3 tumours with sequence alterations in intron 5 (EVMU, MACA, MEMcG), one of which also had an alteration in intron 1 (ANKE), and at least 3 control tumours with normal hMSH2 sequences (ROPE, SHCH, GEHA, BUCH) was analysed to assess amount and length of hMSH2 transcript. RNA was reverse transcribed and then subjected to three PCR reactions using primers spanning the regions of interest (exon 2, exon 5 and exons 2-5), followed by a quantitative PCR assay using  $\beta$ -globin as an internal control. Results are illustrated in Figure 9.

No difference was detected between hMSH2 transcript length across the three amplified regions in tumour samples with intronic mutations compared with non-mutated controls, suggesting that the common intronic mutations do not cause aberrant splicing of hMSH2. Transcript length across exons 2 and 5 was shown to be normal in all tumours, although amplification of the longer PCR product across exons 2-5 produced a weak larger band in all samples, possibly due to the presence of low levels of an alternatively-transcribed gene product.

Quantitation of the hMSH2 transcript relative to the  $\beta$ -actin transcript yielded surprising results (Figure 9). The 3 control tumours (ROPE, SHCH, GEHA) showed levels of hMSH2 transcript ranging between 97% and 72% of the total reaction product (taking average from reactions done in triplicate). Compared with these tumours, samples with mutations in intron 5 (EVMU, MACA) or both intron 2 and 5 (ANKE) showed lower levels of hMSH2 transcript, ranging from 34% to 57%. However, levels of hMSH2 transcript in 2 normal colonic tissue samples (N1, N2) were even lower, at 21% and 24%. Thus, whilst the amount of hMSH2 transcript present in tumours with intronic hMSH2 mutations appears to be lower than those without, transcript levels in all tumours were higher than in normal colonic tissue.

Repetition of the quantitation reaction on another occasion using the same cDNA yielded consistent results. However, cDNA produced from TRIzol-extracted RNA produced widely variable results (not shown), and was excluded from analysis.

## Figure 9

### Analysis of hMSH2 cDNA.

Agarose gel electrophoresis of cDNA amplification products from xenografted tumours known to contain intronic sequence alterations hMSH2, amplified to check for transcript length changes (A-C) and quantitatively (D).

(A) Amplification of exon 2 from primers within exons 1 and 3. Lanes 1 and 10 contain molecular weight markers  $\phi$ X174 RF DNA/*Hae*III and Marker V (Boehringer-Mannheim Ltd), respectively. Lane 2, blank; lane 3, tumour with mutation in intron 5 (MACA); lane 4, tumour with mutation in intron 1 and intron 5 (ANKE); lanes 5-9, control tumours without intronic mutations (ROPE, GEHA, BUCH, SHCH, ANBA).

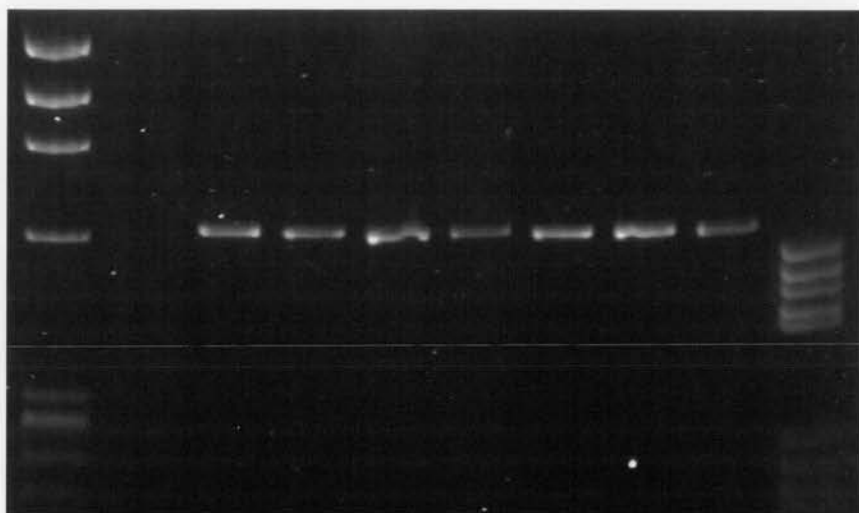
(B) Amplification of exon 5 from primers within exons 4 and 6. Lane 1 contains molecular weight marker 1kb ladder (Life Technologies Ltd). Lanes 2 & 3, tumours with mutations in intron 5 (EVMU & MACA); lane 4, mutant for both introns 2 and 5 (ANKE); lanes 5-9, control tumours without mutations as in (A). Lane 10, Marker V (Boehringer-Mannheim Ltd).

(C) Amplification of exons 2-5 from primers within exons 1 and 6. Lanes 1 & 2, tumours with mutations in intron 5 and introns 1 and 5, respectively (MACA, ANKE); lanes 3-7, control tumours without mutations as in (A). Marker in lane 8 is  $\phi$ X174 RF DNA/*Hae*III.

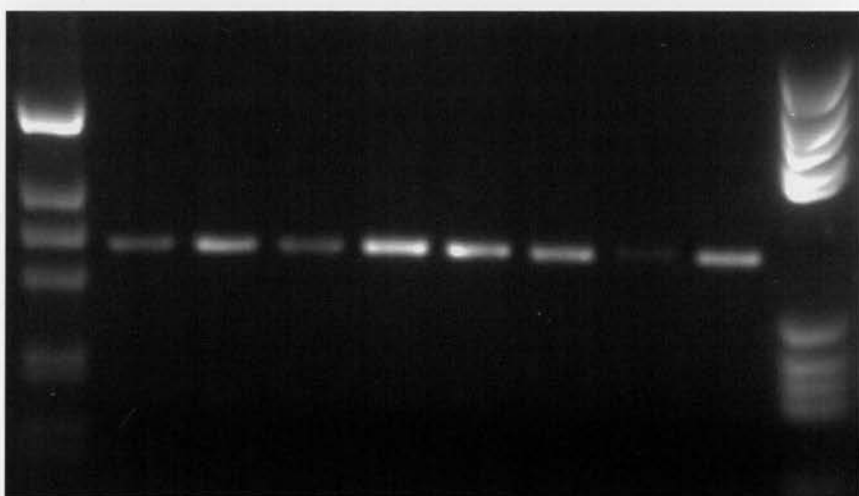
(D) Example of quantitative PCR of hMSH2 cDNA from xenografted tumour samples. The top band is the amplification product of the internal control,  $\beta$ -actin, and the bottom band is the PCR product from hMSH2. Lanes 1-3, control samples (ROPE, SHCH, GEHA) with no hMSH2 mutation; lanes 4-6, tumours with somatic mutations of the hMSH2 intron 5 polyadenine tract (ANKE, EVMU, MACA). Tumour ANKE also has a mutation of the polythymine tract of intron 1. Lanes 7-8, normal colonic tissue. Lane 9, DNA molecular weight marker V (Boehringer Mannheim).

(E) Quantitative analysis of the above experiment, done in triplicate. Bars represent mean hMSH2 product expressed as a percentage of total PCR product (ie hMSH2 plus  $\beta$  actin) over three experiments. Error bars show standard error.

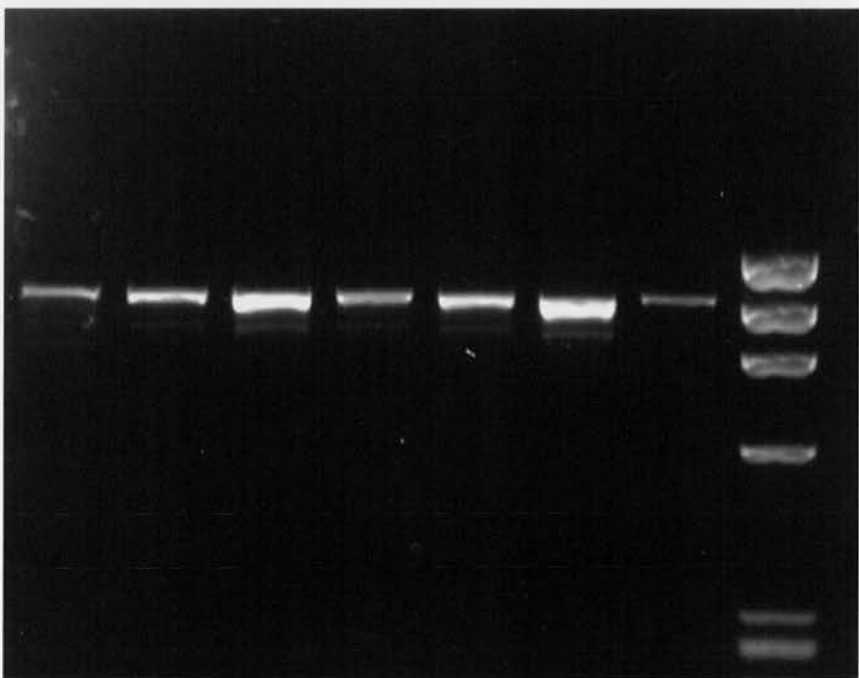
**A**



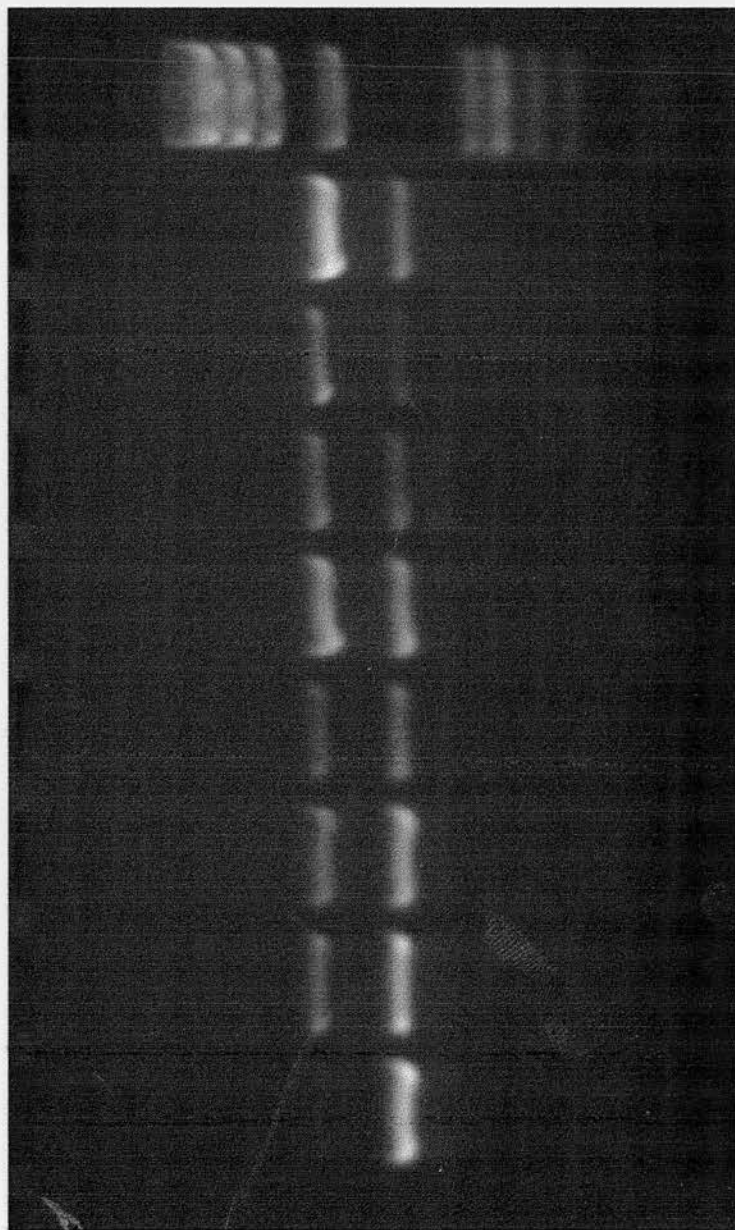
**B**



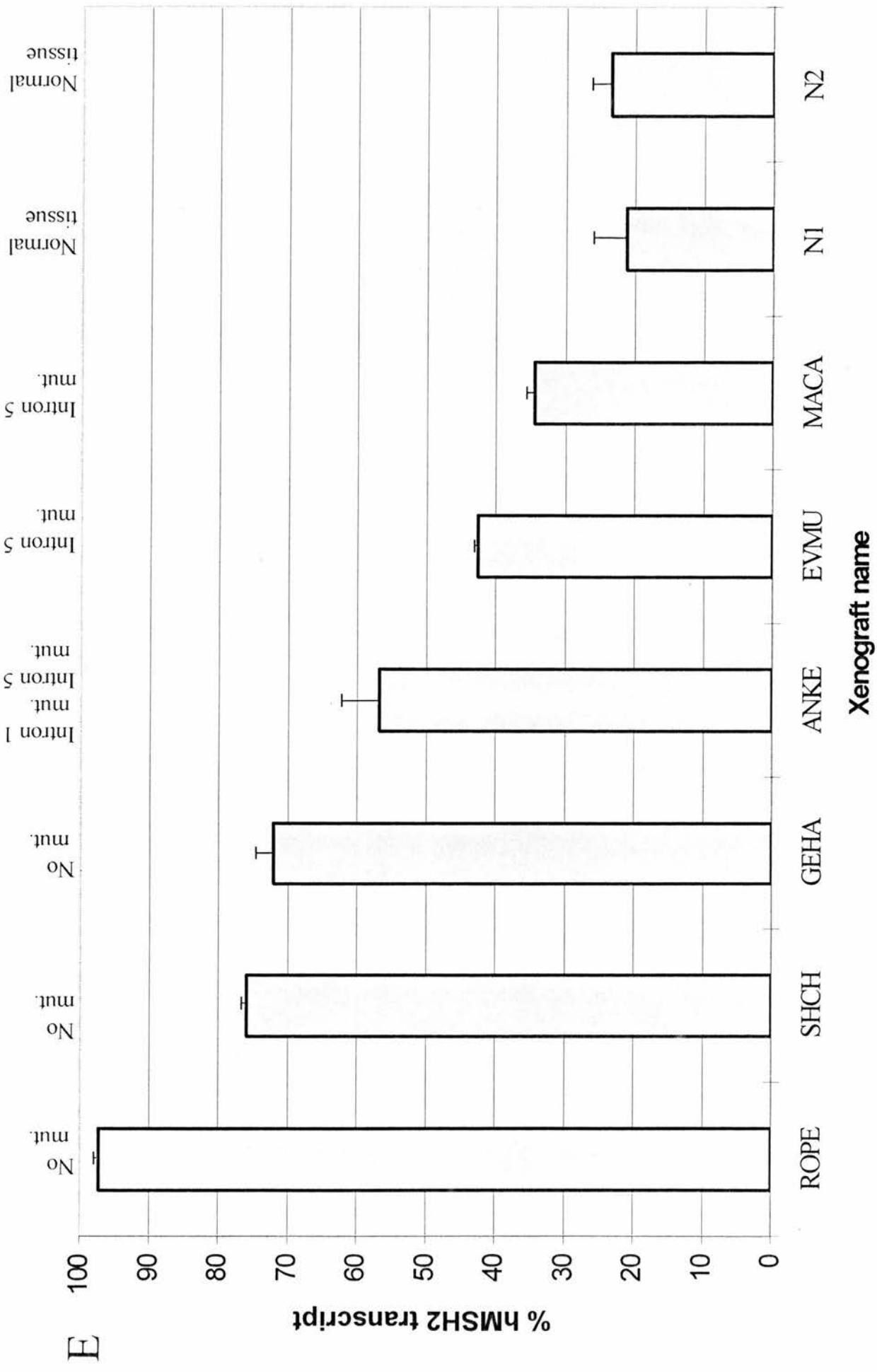
**C**



D







### 3.4. Discussion.

#### 3.4.1 MSI and clinicopathological features of sporadic colorectal cancer

This study has identified a group comprising a sixth of a population of Scottish sporadic colorectal cancer patients that exhibit microsatellite instability. This proportion is in agreement with the incidence of MSI identified by others in sporadic CRC from other geographic locations, including Lothe *et al.* (1993)-[16.5%]; Aaltonen *et al.* (1993) [13%] and Kim *et al.*, 1994a [13%]. This tumour group includes a number of cases where only one locus is altered, a phenomenon which has been previously noted in colorectal cancer (Peltomaki *et al.*, 1993; Lothe *et al.*, 1993) [discussed below].

Other clinico-pathological factors that may distinguish this group from the general population of colorectal cancer patients were examined. Although there was found to be no significant difference between microsatellite unstable and stable tumours for age of onset, Duke's Stage or gender distribution, the data did confirm the proximal location of colonic lesions with MSI, which has been noted by others (Lothe *et al.*, 1993; Kim *et al.*, 1994a; Thibodeau *et al.*, 1993). The results of the Cox analysis indicate a highly significant favourable effect of MSI status on post-operative survival, even after allowing for the effects of age, Duke's stage, sex and tumour side. Paradoxically, histological features generally associated with aggressive tumour behaviour are common in MSI tumours; in this series 41% were mucin-secreting.

The characteristic clinical and pathological profile of tumours with microsatellite instability, common to both sporadic and hereditary cancers, is intriguing. It has been suggested that the survival advantage stems from an enhanced host immune response to cancer cells arising as a result of a high mutation rate of tumour-associated antigens (Kim *et al.*, 1994a); the rate of frameshift mutation at repeated DNA units and the rate of point mutation in cell lines deficient in hMSH2 or hMLH1 is estimated to be about a thousand times greater than that of mismatch repair-proficient cells (Parsons *et al.*, 1993; Bhattacharyya *et al.*, 1994; Shibata *et al.*, 1994). However, a recent study indicates that mismatch repair-deficient cancers often acquire frameshift mutations in the beta 2-microglobulin (beta 2M) gene, which encodes a protein important in antigen recognition in cytotoxic T cell-mediated killing, and are thus more likely to escape immune surveillance (Bicknell *et al.*, 1996). An alternative explanation is that the tumour reaches a situation where it can no longer survive with such a mutation burden if, for example, it compromises the function of essential housekeeping genes. Exactly how the survival advantage comes about remains to be resolved, and it will be of particular interest to discover the means by which host immune response to tumour cells with replication error phenotype is moderated. The abundance of tumours with MSI which secrete mucin might be explained by the notion that such tumours, by retaining the

ability to secrete mucin, retain a more normal phenotype than those which have lost this ability (probably as a result of massive genomic disruption). Alternatively, one consequence of the high mutation rate in cells with defective mismatch repair may be dysregulation of genes controlling mucin production, and hence overproduction of mucin.

The frequent initiation of tumours with unstable microsatellites and characteristic pathology in the proximal colon may indicate that factors present in the local environment favour growth of cells with the characteristics of defective mismatch repair. Although the carcinogenic burden is believed to be higher in the left colon due to slower passage of faecal material, constitutive genetic differences exist between the left and right colon which may increase the concentration of certain mutagens in the proximal colon. For instance, certain polymorphic variants of drug metabolizing enzymes appear to confer susceptibility to colorectal cancer in general, but to proximal lesions in particular. Two such examples are the increased incidence in colorectal cancer patients compared to non cancer-bearing control individuals of a 'slow metabolizing' polymorphism of epoxide hydrolase, an enzyme responsible for detoxification of reactive epoxides (Dr S.Hubbard, personal communication), and of a null mutation of an enzyme which detoxifies polycyclic aromatic hydrocarbon metabolites, glutathione S-transferase mu (GSTM1) (Zhong *et al.*, 1993). Inefficient metabolism of specific mutagens present in the diet, or delayed removal of mutagenic intermediate products of metabolism, may result in particularly high levels of certain carcinogenic substances within the proximal bowel, favouring growth of one or more cells in which a mismatch repair-associated activity is defective. The initial mismatch repair gene mutation may be caused by the carcinogen itself, by other dietary components or by-products of metabolism, or may be already present in the germ-line. Although mismatch repair genes are believed to require homozygous mutation in order to lose repair function, a heterozygous effect of such mutations has been demonstrated *in vitro* (Parsons *et al.*, 1993), and it is possible that such cells maintain growth with a somewhat increased rate of mutation and a level of resistance to damage-induced apoptosis characteristic of cells with such defects. Mismatch repair function is incapacitated when a second allele is affected, after which tumour suppressor genes rapidly acquire mutations and accelerated progression to carcinoma status ensues. Kinzler and Vogelstein (1996) propose that mutations occurring in growth-controlling genes produce conflicting signals within the cell and normally cause the cell to die by apoptosis. However, exposure of the gut to mutagens causes not only mutation but also substantial cell death, and the regeneration which follows gives rise to cells in which the apoptosis-promoting signals are necessarily switched off. They suggest that only in these conditions are cells which acquire, or have already acquired, mutations in growth-controlling genes, susceptible to uncontrolled growth.

Determination of the MSI status of a colorectal lesion could be important not only as a predictor of prognosis but also in terms of potential cancer therapy. Mismatch repair deficiency confers resistance to certain alkylating agents such as N-methyl-N'-nitro-N-nitrosoguanine (MNNG), N-methyl-N-nitrosourea (MNU) and the chemotherapeutic drug temozolomide (Branch *et al.*, 1993 & 1995; Kat *et al.*, 1993; de Wind *et al.*, 1995; Liu *et al.*, 1996b) and, to a lesser extent, to cisplatin (Aebi *et al.*, 1996; Drummond *et al.*, 1996; Fink *et al.*, 1996; Mello *et al.*, 1996). However, such cells retain sensitivity to chloroethylating agents (Liu *et al.*, 1996b). The potential to tailor chemotherapeutic regimens to a tumour's molecular status in order to maximise anti-tumour effect whilst avoiding inappropriate treatment of resistant tumours (which may actually exacerbate the disease process by increasing the cancer's mutational burden) offers real promise of effective drug treatment for a significant number of cancers. Further, mutations induced by mismatch repair deficiency could provide targets for corrective gene therapy (Benson & Wells, 1995). Recent insights into the mechanism of development of tumours with MSI suggest that mutation of an intragenic 'target' polynucleotide repeat within the TGF  $\beta$  receptor subunit II (TGF $\beta$  RII) renders colorectal tumour cells insensitive to TGF $\beta$ -mediated growth inhibition (Markowitz *et al.*, 1995). Mutations at similar repetitive units within the insulin-like growth factor II receptor (IGF-IIR) probably compromise the same pathway of growth inhibition in a subset of mismatch-repair deficient gastrointestinal cancers (Souza *et al.*, 1996), and very frequent mutations of a polynucleotide tract within the apoptosis-promoting gene Bax are predicted to render many cancers with MSI less liable to undergo apoptosis (Rampino *et al.*, 1997). Surprisingly, such mutations are not detected at random; repetitive nucleotide units within many other oncogenes and tumour suppressor genes undergo no such alterations in mismatch repair-deficient cancers (Simms *et al.*, 1997), suggesting a strong selective pressure in favour of mutation of only certain genes critical for inhibition of cancer development.

In addition to the distinct clinical and pathological profiles of this group of tumours, there was a significant inverse relationship between p53 stabilisation and MSI status in tumours with instability at more than one locus (ie those tumours which were RER+). This relationship has also been reported by others (Ionov *et al.*, 1993), although without indication whether the tumours analyzed were sporadic or hereditary in nature. Furthermore, MSI tumours were about twice as common within the group of tumours which had retained both alleles of p53. Thus, the results of this study provide further evidence that distinct genetic defects drive different pathways of colorectal carcinogenesis. A very recent study provides *in vitro* evidence for the existence of two such mechanisms in colorectal cancer, one involving defects in the mismatch repair process with concurrent instability at microsatellite loci and a second leading to extensive chromosomal instability (Lengauer *et al.*, 1997b).

### 3.4.2 The role of hMSH2 in sporadic colorectal cancer

Although sporadic colorectal cancers with MSI demonstrate replication slippage in a similar manner to HNPCC lesions and share similar biological characteristics, there are clear differences. Unlike the situation seen in sporadic colorectal cancer, microsatellite instability occurs as an early event in HNPCC adenomas (Young *et al.*, 1993; Aaltonen *et al.*, 1994), as it does in Barrett's oesophagus, oesophageal adenocarcinoma (Meltzer *et al.*, 1994) and ulcerative colitis-associated lesions (Suzuki *et al.*, 1994). Furthermore, it is clear that the role of hMSH2 is different. Only three percent (1/36) of unstable tumours and 6% (2/32) of stable tumours had one or more mutation in the exonic portions of hMSH2. Thus, this study concurs with the results of the analysis of similar series of sporadic tumours (Børresen *et al.*, 1995; Konishi *et al.*, 1996; Herfarth *et al.*, 1997) and is akin to the incidence of hMSH2 mutation in 20 cell lines derived from sporadic colorectal cancers with MSI (Liu *et al.*, 1995b). However, it is in direct contrast to HNPCC where analysis of these regions identifies a mutation incidence of up to 40% (Liu *et al.*, 1994; Wijnen *et al.*, 1995).

In this series of sporadic cancers, frequent mutations occurred in non-coding regions of hMSH2. All but one of these occurred within two polynucleotide repeat tracts, a polythymine tract of the intron 1 splice donor site and a polyadenine tract repeat within intron 5, suggesting that they may be the effect, rather than the cause, of a generalised mutator phenotype. Although not directly affecting the coding sequence, mutations of this nature, located within or close to splice sites, can cause inefficient or aberrant splicing (Wieringa *et al.*, 1984; Chu *et al.*, 1993). However, the great majority of hMSH2 mutations were in this class; it would be surprising, if hMSH2 were critical for microsatellite instability in sporadic cancer, for the predominant mechanism of inactivation to be one that does not observed in HNPCC. Moreover, analysis of RNA from such tumours suggests that these sequence alterations are unlikely to exert a profound effect upon hMSH2 transcription, since lesions with and without such intronic mutations showed that only normal length hMSH2 transcript was detectable. A certain amount of caution must be exercised in interpreting these data, since, in some circumstances, incorrectly spliced mRNA can be degraded more rapidly than normal (for review see Beelman & Parker, 1995) and could remain undetected. Results of quantitative RNA analysis, though inconclusive, did suggest a possible effect of such mutations on transcription, as the amount of hMSH2 transcript was somewhat lower in tumours carrying such intronic mutations compared to those without. Difficulties with reproducibility of quantitative PCR, discussed in 3.3.3.3, probably mean that collection of new samples (primary or xenografted tumour) would be necessary for reliable confirmation of these data by Northern analysis. In addition, it is clear that a larger number of tumours needs to be studied. All tumours exhibited a level of hMSH2 transcript higher than that of normal



tissue, suggesting that the gene could be up-regulated in colonic tumours. This may be due to the increased rate of cell division in tumour tissue compared with normal, since levels of hMSH2 have been shown to be higher during active stages of the cell cycle compared with resting cells (Marra *et al.*, 1996).

In this study, a proportion of hMSH2 mutations may have escaped detection as SSCP analysis cannot detect all mutations, although the efficacy of the technique for detecting point mutations and small insertions and deletions has been demonstrated in our laboratory in the study of other genes (Curtis *et al.*, 1994; Morris *et al.*, 1996). Mutations in the promotor region, large losses or methylation changes could not have been detected by this method. Perhaps this indicates inactivation of the gene by a mutation not detectable by the methods used in this study, or by another mechanism altogether. However, despite limitations of mutation detection, extensive examination of the known components of the mismatch repair system, hMSH2, hMLH1, hPMS1, hPMS2 and GTBP, by several laboratories using diverse methods have yielded few gene mutations or protein malfunctions. GTBP and hMLH1 mutations do account for microsatellite instability in several cases of sporadic cancer (Papadopoulos *et al.*, 1995; Malkhosyan *et al.*, 1996b; Herfarth *et al.*, 1997) and errors of DNA replication itself are at fault in a small proportion of cases; a small number of mutations in the proof-reading domain of polymerase  $\delta$  have been identified in sporadic cancers (da Costa *et al.*, 1995). The cause of the mutator phenotype in sporadic cancers could be another, as yet unidentified, component of the mismatch repair pathway; no homologue of the *E. coli* *MutH* gene has yet been identified, for example. Another possibility could be a gene product such as proliferating cell nuclear antigen (PCNA), a protein which enhances the processivity of pol $\delta$  and pol $\epsilon$  and is essential for replication. In *S. cerevisiae*, PCNA has been directly implicated in mismatch repair, and missense mutations of *POL30*, the gene encoding PCNA, can give rise to destabilisation of a GT repeat (Johnson *et al.*, 1996b). It is of interest that a mutation of hMSH3 has been detected in endometrial cancer with microsatellite instability (Risinger *et al.*, 1996); this gene could be important in the genesis of a similar phenotype in sporadic colorectal cancer. Given the identical mutator phenotype and shared clinico-pathological features of HNPCC and sporadic cancers with microsatellite instability, it seems unusual that the putative defect in sporadic disease accounts for virtually no cases of HNPCC (since almost all are caused by defects in hMSH2, hMLH1, hPMS1 and hPMS2). This might suggest that the defect(s) occurring in sporadic cancer result in embryonic lethality when present in the germ-line. Although this appears unlikely in view of the relatively normal development of mice totally deficient in *Msh2*, *Pms1* and *Mlh1* (Baker *et al.*, 1995 & 1996; Reitmair *et al.*, 1995; de Wind *et al.*, 1995), the component may be one which is also fundamental to other cellular processes, for instance a helicase, a polymerase or PCNA. The fact that sporadic endometrial adenocarcinomas (which also occur commonly in HNPCC

patients) displaying microsatellite instability also harbour very few mutations in the mismatch repair genes hMSH2, hMLH1, hPMS1 and hPMS2 (Katabuchi *et al.*, 1995; Kowalski *et al.*, 1997) supports the assumption that the major cause of microsatellite instability in sporadic cancer remains to be determined.

The overall incidence of hMSH2 mutation in this population of sporadic colorectal cancers is at least 11%, with only 1% having exonic mutations. Amongst this population of patients with apparently sporadic colorectal cancer, three carried germ-line mutations in hMSH2. This small subset of patients may be thought to represent hereditary, rather than sporadic, colorectal cancers which do not fit the criteria for HNPCC. Only one of these 3 patients had any family history of cancer. Interestingly, this patient was identified from the group of cancers without MSI. He was younger (49 years) than the average age-group, had a Duke's C carcinoma in the ascending colon, and had a family history of pancreatic cancer. This individual may belong to the class of familial RER+ tumours including lung, breast and pancreas, with less prevalent microsatellite alterations (Liu *et al.*, 1995b) although this has not previously been associated with hMSH2 mutation. A second patient with no family history or evidence of microsatellite instability, aged 74 and with a cancer of the caecum, was also found to have a germ-line hMSH2 mutation. Both of these mutations were mis-sense and therefore predicted to compromise protein function. The third germline mutation was the shortened polyadenine tract in intron 5; found in a patient whose tumour demonstrated MSI. These observations support the view that a wide range of phenotypes are associated with germ-line hMSH2 mutation (Kolodner *et al.*, 1994; Mary *et al.*, 1994).

### 3.5. Conclusions

Two major conclusions can be drawn from this work. Firstly, although MSI is clearly a significant factor in the clonal expansion of around one in six of all sporadic colorectal cancers, exonic hMSH2 defects are associated with this instability in a very much lower proportion of tumours than in HNPCC. Indeed, when mutations in the intronic sequences of hMSH2 are excluded from consideration, hMSH2 mutation in sporadic tumours with MSI is no more frequent than in other tumours. Therefore, it appears that the genetic basis of microsatellite instability in sporadic and inherited colorectal cancer is different. Secondly, the data (from a large series of patients with extensive follow-up) clearly demonstrate a survival advantage for patients who have a tumour exhibiting MSI. It will be of great interest to determine the mechanism by which such genetic instability imparts improved prognosis since this may have substantial implications for novel therapeutic approaches.



## CHAPTER 4

### TEMPORAL EVOLUTION OF MICROSATELLITE INSTABILITY IN SPORADIC COLORECTAL CANCER XENOGRAFTS

#### 4.1. Introduction

Cells in which mismatch repair is defective exhibit high mutation rates at microsatellite loci. *In vitro* studies show that cell lines derived from colorectal tumours with microsatellite instability continue to generate de novo mutations in di- and trinucleotide repeat sequences at each round of replication at a rate of  $5 \times 10^{-4}$  to  $10^{-2}$  per allele per cell replication (Shibata *et al.*, 1994). Furthermore, primary cancers with unstable microsatellites usually (though not always) display heterogeneity of novel microsatellite alleles throughout the tumour, suggesting that the generation of new mutations often persists throughout tumour growth *in vivo* (Shibata *et al.*, 1994). This study aimed to test directly whether the replication error phenotype persists throughout prolonged carcinoma growth *in vivo* through analysis of microsatellite loci in a series of human colorectal tumours growing as xenografts.

It is not known whether MSI can be acquired spontaneously during carcinoma growth. The very low incidence of MSI in sporadic colorectal adenomas compared with carcinomas (Aaltonen *et al.*, 1994; Young *et al.*, 1993) implies that it might be associated with the transition from adenoma to carcinoma, but it could alternatively be a feature of a carcinoma type which is not preceded by an adenomatous stage. MSI does arise, however, in conjunction with drug resistance in cancer cell lines challenged with cisplatin or doxorubicin (Anthoney *et al.*, 1996). Acquisition of such a mutator phenotype at a later stage in a real tumour could have implications for tumour management, as MSI is associated with sensitivity to certain chemotherapeutic drugs but resistance to others (discussed in Chapter 2, section 2.5). Thus, a further aim of this study was to examine replication-competent sporadic colorectal carcinomas over time in order to determine whether the mutator phenotype can arise spontaneously.

*In vivo* study of growing human tumour tissue is only possible through subcutaneous implantation as xenografts into SCID mice. Such tumours retain remarkably similar pathological and genetic features to the primary tumour, including histological pattern (McQueen *et al.*, 1991), deletion or mutation status of p53, DCC, APC and Ki-ras (Lefrançois *et al.*, 1989; McQueen *et al.*, 1991; Ms S. White, personal communication), stability of p53 by immunohistochemistry (Ms S. White, personal communication), DNA ploidy (Ms S. White,

personal communication) and karyotype (Lefrançois *et al.*, 1989), although an increased tendency towards polyploidisation has been noted. To date, the behaviour of microsatellite loci in these tumours has not been studied.

## 4.2. Materials and methods

### 4.2.1 Outline of methods

Xenografts were established and harvested, and DNA extracted from normal, primary tumour, early xenograft and late xenograft tissue samples as described in 2.2-2.3. Samples were deemed to be early passage when they had been passed through not more than 3 mice and late passage when they had been passed through at least four mice (approximately 4 months). Tumours were assessed for instability using a PCR/silver staining method at four microsatellite loci, D2S123, D3S1293, D8S282 and D13S160 as described in 2.7.1-2.7.2.

Initially, 33 xenografted tumours were available for assessment of microsatellite instability status at late passage (see Appendix 5). All but 2 were also available for assessment at early passage, and in two others, primary tumour tissue was not available. A further five tumours were available only at early passage. In most cases, tumours were examined after 1 and 6 passes. Tumours in which microsatellite instability occurred underwent DNA fingerprinting by at least one of two methods, Southern analysis of a variable number tandem repeat (VNTR) or PCR-based analysis of an *Alu* repeat, to ensure that no sample mixup had occurred.

### 4.3. Results

Thirty-three xenografted tumours from which late passage tumour DNA was available were initially included in this study. All were examined at the earliest available passage (usually pass 1, occasionally pass 2 or 3) and the latest available passage (pass 4 to 7, and passes 18 and 23 in one case). Examples are illustrated in Figure 10. Nine demonstrated microsatellite instability at one or more loci, although four were unusual, demonstrating apparent instability in the xenografted tumours but not the corresponding primary tumour (in one case only in the late passage xenograft) suggesting the onset of instability during xenograft progression. DNA fingerprinting of all unstable tumours demonstrated the presence of novel alleles at two different regions (one VNTR and one *Alu* repeat sequence) in these 4 samples when compared with the DNA from their matching normal tissue (Figure 11) and thus excluded these samples from the series. The other 5 tumours with MSI showed concordance between normal and tumour tissue on fingerprinting. Thus, a total of 5 out of 29 (17%) xenografted tumours included in the assessment of microsatellite instability at late passage demonstrated microsatellite instability. Details of allele shifts and passage numbers for these tumours are shown in Table 12. In all 5 cases the instability was apparent in both primary tumour and xenograft samples. Instability was always apparent at 2 or more loci, therefore all could be termed RER+ (see 1.3.11.1).

None of 24 xenografted tumours whose primary cancer had stable microsatellite sequences developed MSI, even, in one case, where the tumour had been passed 23 times through mice over a period of approximately 2 years.

A further 6 tumours (including one which was excluded from the original series because fingerprinting demonstrated the late passage xenograft to be erroneous) were only available for study at early passage. Data from these tumours were in keeping with that of the main series of cancers; 4 demonstrated stability in the primary tumour and xenograft, whilst 2 showed instability in the primary tumour and xenograft, one at one microsatellite locus and one at all 4 loci (Table 12).

Microsatellite allele shift did not occur within every locus at every pass. Novel alleles, of different lengths to those in normal tissue, demonstrated subsequent stable transmission at up to three loci in 5 of the 7 xenografted tumours with MSI (Table 12). The majority of mutations at microsatellites were 2 or 4 nucleotide insertions or deletions although changes of up to 12 nucleotides were observed in two tumours (ANKE and MEMCG late passage xenografts).

Table 12

Allele shifts at 4 microsatellite loci in sporadic colorectal primary tumours and xenografts with microsatellite instability.

LOCUS											
Xenograft pass no.			D2S123			D3S1293			D8S282		
Xenograft name	Early	Late	Tum.	Early	Late	Tum.	Early	Late	Tum.	Early	Late
ANKE	1	6	O	O	O	↑	↑	↑	O	↑	↑
EVMU	1	5	↑	↑	↑	O	↑	↑	↑	↑	↑
LADI	1	5	↑	↑	↑	O	O	O	O	↑	↑
MEMCG	1	4	O	↑	↑	↑	↑	↑	↑	↑	↑
MACA	1	4	O	↑	↑	O	O	↑	↑	↑	↑
CODR	1	na	O	O	na	↑	↑	na	O	O	na
JEGA	3	na	O	↑	na	O	↑	na	↑	↑	na

O indicates no shift in allele size from normal

↑ indicates a shift in allele size from normal

↑↑ indicates a second shift in allele size from normal

↑↑↑ indicates a third shift in allele size from normal

na = not available.

Tum. =primary tumour, Early=early xenograft, Late=late xenograft.

## **Figure 10**

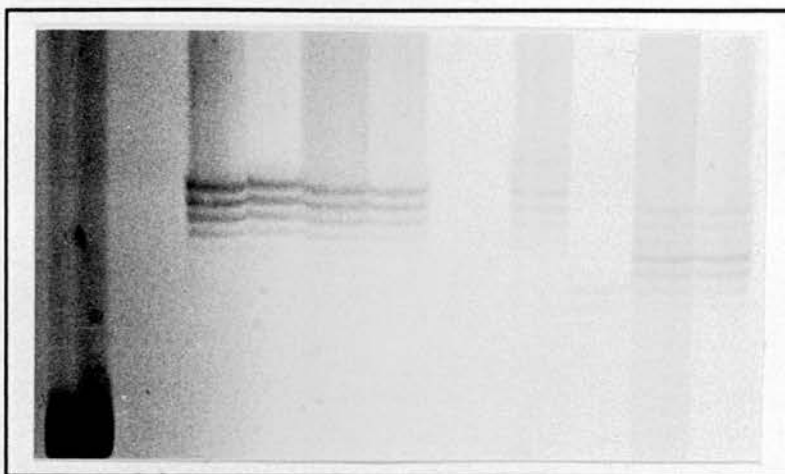
### **Microsatellite instability in xenografted colorectal carcinomas**

Silver stained polyacrylamide gel analysis of PCR products generated from four microsatellite loci, (A) D13S160, (B) D8S282, (C) D3S1293 and (D) D2S123, in normal (N), primary tumour (T), early xenograft (XE) and late xenograft (XL) tissue from two patients, GEHA and MEMcG. GEHA demonstrates no microsatellite allele shifts, whereas MEMcG shows instability at all four loci. Early xenografts were at passage number 1 for both GEHA and MEMcG, late xenografts at passage 6 and 4, respectively.

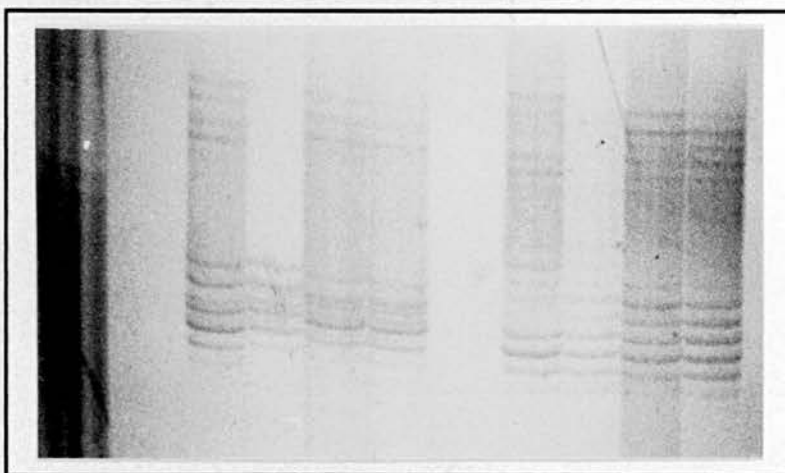
M=1kb ladder DNA molecular weight marker (Life Technologies, Ltd).



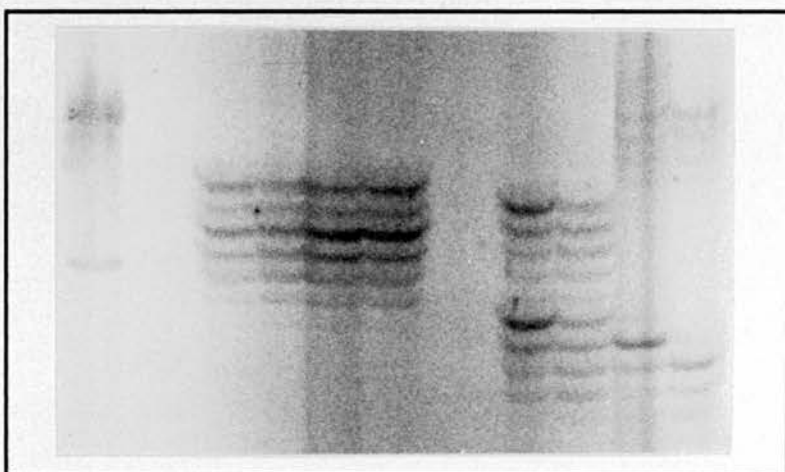
A



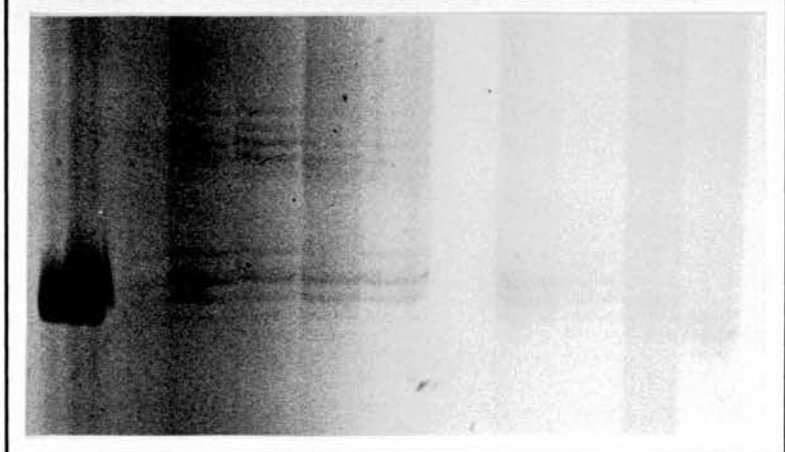
B



C



D



M

N

T

XE

XL

— GEHA —

N

T

XE

XL

— MEMCG —

## Figure 11

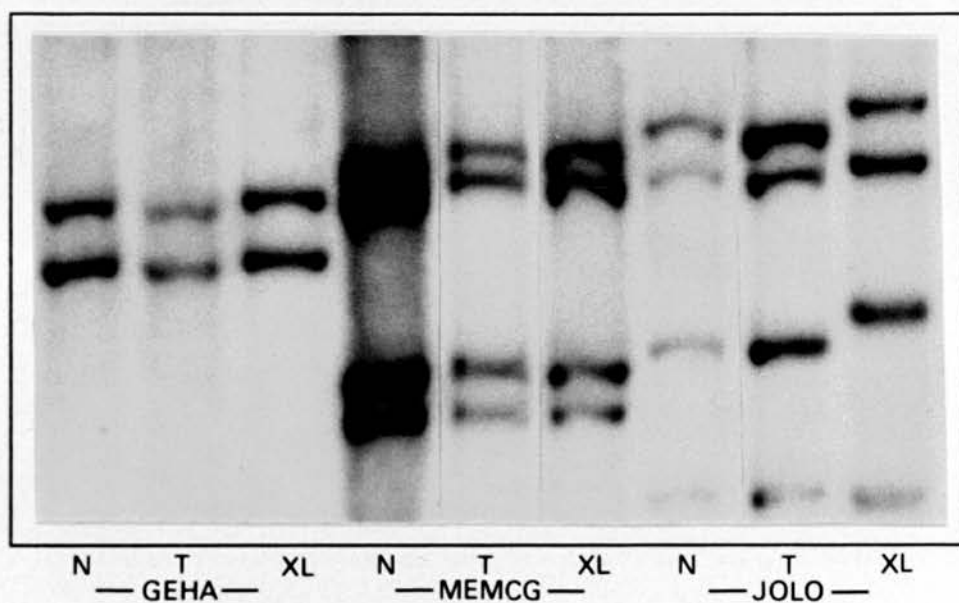
### DNA fingerprinting of xenografted tumour samples

(A) Southern analysis with probe 29C1 (Cooke *et al.*, 1985) of normal (N), primary tumour (T) and late xenograft (XL) material from three individuals, GEHA, MEMcG and JOLO. GEHA and MEMcG show the same allele pattern in all samples analysed. JOLO contains novel bands in late xenograft tissue compared with the corresponding normal and primary tumour, indicating a likely sample mixup. This sample was excluded from the series.

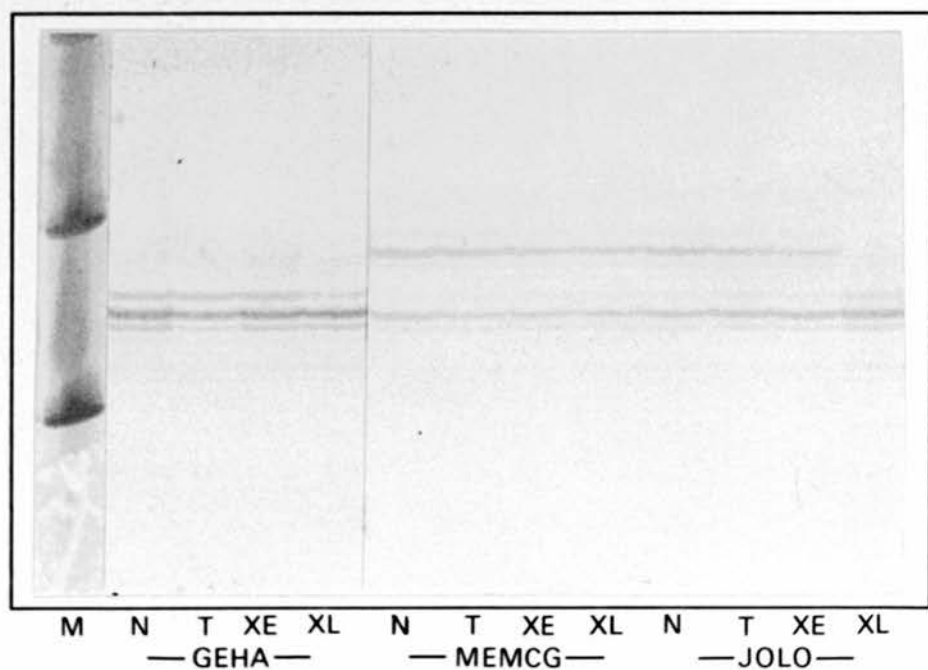
(B) The same samples analysed by PCR amplification of an *Alu* repeat within the p53 gene. Again, GEHA and MEMcG show concordant banding patterns, whereas JOLO shows loss of A1 in XL.

M=1kb ladder DNA molecular weight marker (Life Technologies, Ltd).

A



B



#### 4.4. Discussion and Conclusions

The data presented here demonstrate that the replication error phenotype of sporadic colorectal tumours grown as xenografts is maintained over a prolonged period of time. New microsatellite alleles arise continually during growth (though not at every locus with every passage), consistent with the presence of underlying defects in the process of mismatch repair rather than a transient mutational event at the onset of carcinoma. This is concordant with the work of Shibata *et al.* (1994) who demonstrated maintenance of this phenotype *in vitro* and evidence suggestive of its maintenance *in vivo*. Additionally, a recent study used microdissection of RER+ gastric tumours to demonstrate intratumoral clonal heterogeneity of microsatellites, suggesting that evolution of such sequences can occur *in vivo* (Ottini *et al.*, 1997). Similarly, primary cancers in which microsatellite sequences were stable, maintained replication fidelity when grown as xenografts over an extensive period (growth for up to 2 years in one case), demonstrating that spontaneous onset of the replication error phenotype during cancer progression is a rare event. Thus, the data suggest that the mutator status of a colorectal tumour, as assessed after surgical resection, is fixed.

These data are compatible with the current hypothesis that the mutator phenotype represents one of at least two tumorigenic processes responsible for the development of colorectal cancer (Kinzler & Vogelstein, 1996; Lengauer *et al.*, 1997b). Lengauer *et al.* (1997b) have demonstrated a second mutagenic process in cell lines derived from colorectal cancer in which spontaneous chromosomal anomalies occur very frequently (termed CIN, for chromosomal instability). Although the two mutagenic pathways can coexist, the authors believe that only one is required for cancer progression and the CIN phenotype is dominant. Conceivably, the stable cancers in this series do spontaneously generate microsatellite instability but growth of such cells is not favoured, perhaps due to the preexistence of a potent alternative tumorigenic pathway. The *in vitro* acquisition of a mutator phenotype in response to cisplatin and doxorubicin (Anthony *et al.*, 1996) implies that certain circumstances do favour growth of clones with replication errors. An investigation of the effects of several anti-cancer drugs on selected tumours from this series is currently underway and should yield interesting results.

The results of this study provide an additional demonstration of the value of xenografts as tools for the study of colorectal cancer. The status of replication fidelity at microsatellite sequences can be added to the many genetic and histological features which remain constant during xenograft growth, further confirming these tumours to be legitimate representations of the primary colorectal carcinoma from which they arise. In addition, examination of microsatellite loci through serial passage has shown that, at least with the 4 loci used in this series, characterisation of the primary tumour alone is not always sufficient

for accurate classification as RER- and RER+. Aaltonen *et al.* (1993) originally divided tumours according to whether they were unstable at one (classed as RER-) or at more than one (RER+) microsatellite, and found only the latter group to be associated with certain distinctive clinical and pathological characteristics. These criteria have subsequently been widely, though arbitrarily, employed, since the specificity and number of microsatellites used to define this status varies. In this series, data from the primary tumour alone would have resulted in misclassification as RER- of one tumour (JEGA), in which underlying instability was clearly present. Conversely, the classification employed early in the discovery of this phenomenon (see Chapter 2), in which a tumour with instability at any locus was deemed unstable, would have resulted in misclassification as unstable of another tumour (CODR), which remains stable throughout passage. The allele shift observed on only one occasion in this tumour may be explained by the suggested low level of background microsatellite instability thought to occur in certain repeat types (Weber *et al.*, 1993). In the light of these results, it seems preferable to choose the cautious classification suggested by Aaltonen *et al.* in order to avoid false assignment of essentially stable tumours to the small group of lesions which have intrinsically unstable microsatellites. In future, correct classification might be better achieved by examination of a larger number of selected microsatellite loci, as suggested by recent studies aimed at standardising the assessment of MSI (Bocker *et al.*, 1997; Dietmaier *et al.*, 1997).

## CHAPTER 5

### CHROMOSOMAL INSTABILITY IN SPORADIC COLORECTAL CANCER

#### 5.1. Introduction

Cytogenetic and comparative genomic hybridisation (CGH) studies have determined a number of specific chromosomal abnormalities which occur very frequently in colorectal cancer (Reichmann *et al.*, 1981; Muleris *et al.* 1988 & 1990; Yaseen *et al.*, 1990; Konstantinova *et al.*, 1991; Bardi *et al.*, 1993b; Barletta *et al.*, 1993; Herbergs *et al.*, 1994; Gerdes *et al.*, 1995; Schlegel *et al.*, 1995; Ried *et al.*, 1996). However, recent evidence indicates that at least one of the distinct mechanisms of genomic instability which drive sporadic colorectal carcinogenesis might not necessarily result in chromosomal instability. This mechanism is associated with generalised microsatellite instability caused by defects in the process of mismatch repair and is associated with distinct clinico-pathological features (discussed in Chapter 2). Several studies have demonstrated that in such tumours there is seldom evidence for major abnormalities in chromosome structure or number (Aaltonen *et al.*, 1993; Lothe *et al.*, 1993; Shibata *et al.*, 1994; Remvikos *et al.*, 1995; Branch *et al.*, 1995; Schlegel *et al.*, 1995). A second putative mechanism, which is a feature of the large majority of sporadic colorectal carcinomas, is characterised by defects of p53 function. Numerous studies link p53 defects with chromosomal abnormalities (Bischoff *et al.*, 1990; Livingstone *et al.*, 1992; Yin *et al.*, 1992; Carder *et al.*, 1993 & 1995; Agapova *et al.*, 1996; Remvikos *et al.*, 1997) and, furthermore, disruption of p53 function has been shown to precede aneuploid clonal divergence in colorectal cancer (Carder *et al.*, 1993 & 1995). Several reports suggest an inverse correlation between aberrant p53 function and the microsatellite instability phenotype (Ionov *et al.*, 1993; Kim *et al.*, 1994a; Cottu *et al.*, 1997; Radford *et al.*, 1997; Remvikos *et al.*, 1997), implying that these two abnormalities represent independent tumorigenic mechanisms. *In vitro* evidence also suggests that high levels of chromosomal instability and microsatellite instability are features of independent tumorigenic mechanisms in colorectal cancer (Lengauer *et al.*, 1997b). The latter study suggests that the two processes are not mutually exclusive, though either one is sufficient to drive carcinogenesis.

This investigation set out to determine the extent of chromosomal abnormalities in relation to defects of p53 and microsatellite instability in sporadic colorectal cancer. CGH (Kallioniemi *et al.*, 1992; du Manoir *et al.*, 1993) was used to evaluate chromosomal copy number abnormalities in a series of such tumours, and flow cytometry was employed to determine DNA ploidy abnormalities. Further to this, patterns of chromosomal abnormalities



were examined in order to identify specific common regions of amplification or deletion, which may indicate the presence of an oncogene or tumour suppressor gene, associated with defects of either putative tumorigenic pathway. The study employed tumours grown through one or two passages as subcutaneous xenografts in SCID mice, which are advantageous in their elimination of human stromal contamination from tumour material. As noted in Chapter 4, tumours grown through serial passage in this way display a remarkable adherence to many genetic (S.White, unpublished data; Lefrançois *et al.*, 1989; McQueen *et al.*, 1991) and histological features (S.White, unpublished data; McQueen *et al.*, 1991) of the primary tumour from which they were established, although a degree of karyotypic evolution does occur (Lefrançois *et al.*, 1989). The results of analysis of DNA ploidy, p53 status and chromosomal changes presented here shed further light on the value of xenografted tumours as study models for colorectal cancer.

## 5.2. Material and methods

### 5.2.1. Comparative Genomic Hybridisation

CGH detects changes in copy number of whole or partial chromosomes (Kallioniemi *et al.*, 1992; du Manoir *et al.*, 1993) through simultaneous hybridisation onto a normal metaphase spread of DNA derived from normal and test tissue, differentiated by fluorescent coloured labels. A fluorescence ratio is plotted along the length of each chromosome and any regions of amplification or deletion are detected as a change in ratio of green to red fluorescence. Hybridisation on to normal metaphases makes the technique particularly suitable for analysis of tumour genomes, since good quality metaphases for use with conventional cytogenetic techniques are notoriously difficult to obtain. In addition, as it provides information about the whole genome at once it is considerably more powerful than conventional deletion/amplification study methods which are limited to single loci. However, though it provides an excellent method for detection of amplification and deletion relative to the entire DNA content of the test sample, it cannot detect translocations or pure polyploidisation.

In this study, test DNA was extracted from 29 cancers, 28 derived from primary colorectal cancers and one from a lymph node metastasis from a patient with colorectal cancer, all of which had been grown as xenografts in SCID mice (2.1-2.2) through one or two passages (approximately one or two months) before analysis. The latter tumour was excluded from statistical analysis. Clinical and pathological details for these tumours are presented in Table 13.

CGH was carried out according to the protocol described in 2.13. Tumour DNA was labelled with biotin by nick translation and counter-hybridised against digoxigenin-labelled normal DNA and Cot-1 DNA onto a normal male metaphase spread for 2-3 days. A rhodamine-conjugated anti-digoxigenin antibody and fluorescein-conjugated avidin were used to visualise hybridised normal and tumour DNA. Each experiment included a normal control hybridisation consisting of normal male DNA labelled with biotin vs normal male DNA labelled with digoxigenin. Hybridisations were analysed using the Apple MacIntosh-based quantitative image analysis software MacProbe (Perceptive Scientific Instruments, Ltd). Between five and ten metaphase spreads, or a minimum of 5 autosomes and 3 sex chromosomes, were analysed for each tumour. Green/red ratio cut-off points of 1.125 and 0.875 were chosen for scoring of chromosome copy number changes. This was the level at which copy number changes could easily be visualised, and is equivalent to a loss or gain of one chromosome in 25% of cells in a diploid karyotype. A chromosome arm was scored 'lost' or 'gained' if any region within it (other than excluded areas) reached the appropriate cut-off point, and scored as both 'lost and gained' if both cut-off points were reached.

### 5.2.2 Analysis of p53 status and microsatellite instability

Immunohistochemical staining of p53 was carried out on all primary and 14 xenograft tumour sections as in 2.6 using two antibodies, pAb1801 and DO7, both of which recognize both normal and mutant forms of the protein. Immunohistochemistry (IHC) using these antibodies in a series of tumours often yields a wide inter-tumoral variation in the number and intensity of positive nuclei. Generally, there is an excellent correlation between positive staining with DO7 in  $\geq 30\%$  of nuclei and mutation within the p53 gene (Baas *et al.*, 1994). However, some tumours containing less than 30% positive nuclei also carry a mutation in the p53 gene. Negative IHC usually indicates normal p53 function, but a subset of tumours with mutations of p53, usually either nonsense mutations or point mutations within exon 6, are associated with lack of protein stabilisation (Cripps *et al.*, 1992; Remvikos *et al.*, 1997). Thus, in order to clarify the status of p53 in tumours in which numbers of IHC-positive nuclei were low or negative, SSCP mutation analysis of the gene was carried out (see 2.11 for method). Analysis was restricted to the mutational 'hotspot' region of p53, exons 5-9, where the vast majority of mutations occur (Levine *et al.*, 1991). pAb1801 often produced lower levels of staining relative to DO7, and so low level staining with this antibody was not considered to be sufficient to justify classification as a low-level staining tumour.

Microsatellite instability was assessed using PCR, polyacrylamide gel electrophoresis and silver staining (2.7) in the same manner as in Chapters 3 & 4.

### 5.2.3 Analysis of DNA ploidy

Because CGH cannot detect polyploidisation, DNA ploidy was assessed by flow cytometry (see 2.5). Tissue was available for flow cytometry from 26 tumour samples. Where available (in 22 cases) both primary tumour and xenograft samples were analysed. Three tumours were only available as primary tumours and one only as xenograft material. Aneuploid tumours were assigned an arbitrary status of hyperdiploid or hypotetraploid according to the proximity of the peak to the predicted diploid and tetraploid position.

## 5.3. Results

### 5.3.1 Assessment of p53 status

p53 immunohistochemistry status was assessed in 28 primary sporadic colorectal cancers and one lymph node metastasis from which the early passage xenografts samples in this study derived. Of these, 21 (including the lymph node) stained positive for stabilised p53 protein with pAb1801, DO7 or both, and 8 did not stain with either antibody (see examples in Figure 4). The percentage of immunohistochemically positive nuclei per positive tumour varied from <1% to >90%. This large variation in staining level is well reported, and is strongly associated with mutation of the gene when  $\geq 30\%$  or more of cells stain positive (Baas *et al.*, 1994; Midgley *et al.*, 1992). Fourteen xenografted tumours were also assessed for p53 status by IHC, producing no discrepancies with the primary tumour results.

Of 8 IHC-negative tumours and 11 tumours with low to moderate levels of IHC staining which were assessed for mutation within exons 5-8 of p53, three showed evidence for mutation. Two of these, samples CORU and ELWI, which showed evidence for mutation in exon 5 and exon 6, respectively, were immunocytochemically negative for p53 with both DO7 and pAb1801. The remaining lesion, MACA, with a mutation in exon 8, demonstrated a low level of staining with DO7 but was not assessed for 1801. Thus, taking both IHC and mutation analysis together, a total of 23 tumours (including the lymph node metastasis) showed evidence for defective p53 function. p53 status and clinico-pathological features for these tumours are shown in Table 13.

p53 status, defined by IHC and mutation status, did not appear to define a group of cancers with clear-cut clinico-pathological characteristics; Dukes' stage and tumour side were not significantly different in p53 defective tumours when compared with those in which p53 was apparently normal ( $\chi^2 = 0.173$  and  $p = 1.00$ , respectively).

### 5.3.2 Assessment of microsatellite instability status

Six tumours demonstrated microsatellite instability at 2 or more loci, and were thus termed RER+. Of these, five showed shifts in allele size at 2 or more loci in the primary tumour and one showed instability at only one locus in the primary tumour, but demonstrated widespread shifts in the early passage xenograft (tumour JEGA, see Chapter 4 for details of microsatellite instability in this tumour). A further tumour showed a shift in allele size at one locus in the primary tumour, but demonstrated no further change at any locus through passage (tumour CODR, see Chapter 4) and was thus classified as RER- (Aaltonen *et al.*, 1993). Results are summarised in Table 13.

The characteristics of the cancers in this series with widespread microsatellite instability conform with the predicted phenotype, discussed in Chapter 2, in that they are all derived from the proximal colon (compared with RER-,  $p = 0.016$ , 2-tailed Fisher's exact test).

The proportions of cancers which were Dukes' stage A or B compared with C/D were similar in RER+ and RER- tumours ( $p= 0.173$ ).

### 5.3.3 Flow cytometry

Of 26 samples assessed for DNA ploidy, 8 were near-diploid and 18 were aneuploid (of which 9 were assigned the arbitrary status hyperdiploid, 7 were designated hypotetraploid or tetraploid, and 2 were known only to be aneuploid). Most were assessed in both the primary tumour and xenograft, with only one discrepancy: passage 1 xenograft ANKE was tetraploid, whilst its primary tumour was diploid, implying that endoreduplication had occurred. This tumour was classified 'unknown' for purposes of statistical analysis. Flow cytometry results are shown in Table 13 and Figure 12.

**Table 13 Clinicopathological, molecular and immunocytochemical data for colorectal cancer xenografts**

Tumour name	MSI status	p53 status	DNA content	Age	Side	Site	Dukes stage	Xeno. pass used for CGH
JEGA	RER+	Defective	Near-diploid	68	Right	Asc	B	3
AGSC	RER+	Defective	Near-diploid	71	Right	Cae	C/D <sup>3</sup>	5
ANKE	RER+	Defective	Near-diploid/tetraploid <sup>4</sup>	82	Right	Asc	B	1
MACA	RER+	Defective	NK	81	Right	Cae	B	1
EVMU	RER+	Normal	Near-diploid	49	Right	Cae	B	1
MEMCG	RER+	Normal	Near-diploid	65	Right	Cae	B	1
ANWO	RER-	Defective	Hyperdiploid	77	Right	Asc	B	1
SAGR	RER-	Defective	Near-diploid	39	Right	Cae	B	1
ROPE	RER-	Defective	Near-diploid	83	Right	Cae	C/D <sup>3</sup>	1
ELWI	RER-	Defective	Hypotetraploid	84	Right	Cae	C/D <sup>3</sup>	4
JAMU2	RER-	Defective	Aneuploid <sup>2</sup>	85	Right	Asc	C/D <sup>3</sup>	1
EDLA	RER-	Defective	Hypotetraploid	48	Right	Asc	C/D <sup>3</sup>	1
BUCH	RER-	Defective	Hyperdiploid	81	Right	Asc	C/D <sup>3</sup>	3
PAMCI	RER-	Defective	Hypotetraploid	65	Left	Rec	C/D <sup>3</sup>	1
THLE	RER-	Defective	Hyperdiploid	66	Left	Rec	C/D <sup>3</sup>	2
JOCR	RER-	Defective	Hyperdiploid	36	Left	Sig	C/D <sup>3</sup>	3
CORU	RER-	Defective	Tetraploid	40	Left	Sig	C/D <sup>3</sup>	1
DUMA	RER-	Defective	Near-diploid	68	Left	Des	B	2
EDDO	RER-	Defective	Hyperdiploid	78	Left	Sig	B	1
GEHA	RER-	Defective	Aneuploid <sup>2</sup>	86	Left	Sig	B	1
JOLO	RER-	Defective	Hyperdiploid	56	Left	Sig	B	2
ANBA	RER-	Defective	NK	61	Left	Sig	C/D <sup>3</sup>	3
CODR	RER-	Defective	Hypotetraploid	40	Left	Rec	C/D <sup>3</sup>	1
CADU	RER-	Defective	Hyperdiploid	95	Left	Sig	B	2
HIDE	RER-	Normal	Hypotetraploid	71	Right	Asc	C/D <sup>3</sup>	1
GRZBO	RER-	Normal	NK	87	Left	NK	A	1
JEDO	RER-	Normal	Hyperdiploid	68	Left	Sig	B	1
WITO	RER-	Normal	Hypotetraploid	76	Left	Sig	A	1
ANSA	RER-	Defective	Hyperdiploid	73	Left <sup>1</sup>	LN	na	2

<sup>1</sup> location of primary cancer

<sup>2</sup> data obtained from Ms J Scheler; further details not available.

<sup>3</sup> See Chapter 2 for discussion of classification of tumours as Dukes' C/D

<sup>4</sup> Primary tumour diploid, passage 1 xenograft tetraploid. This tumour classified 'unknown' for statistical analysis.

Cae = caecum, Asc = ascending colon, Des = descending colon, Sig = sigmoid, Rec = rectum, LN = lymph node.

NK = unknown, na = not applicable.

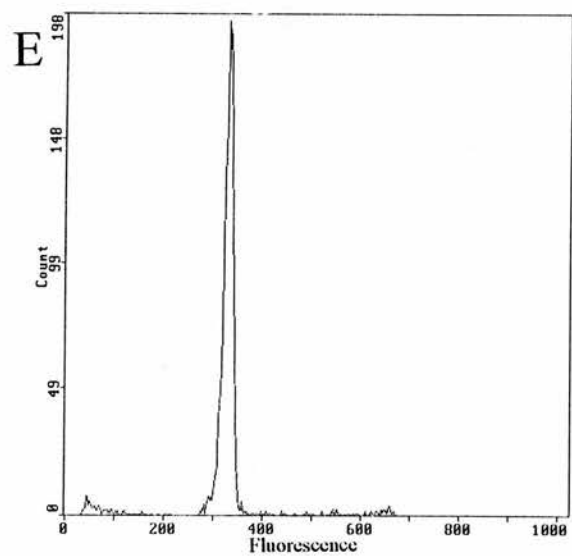
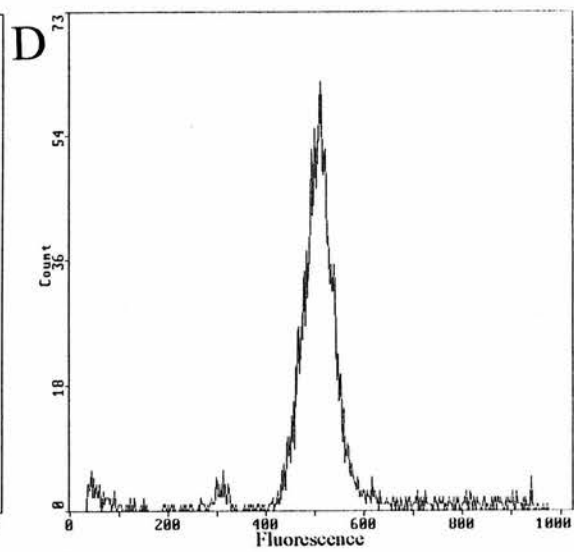
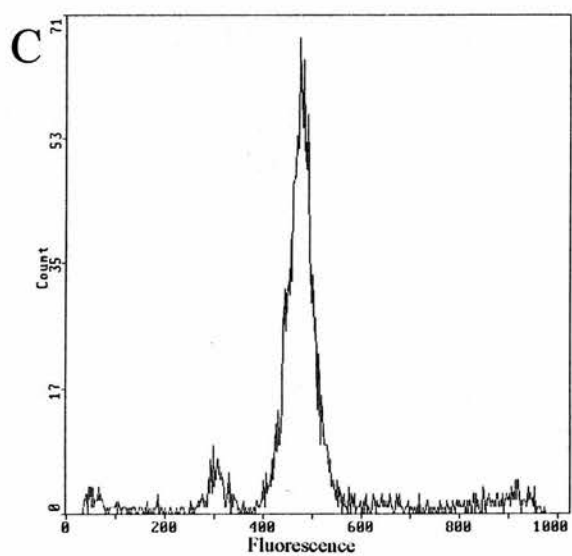
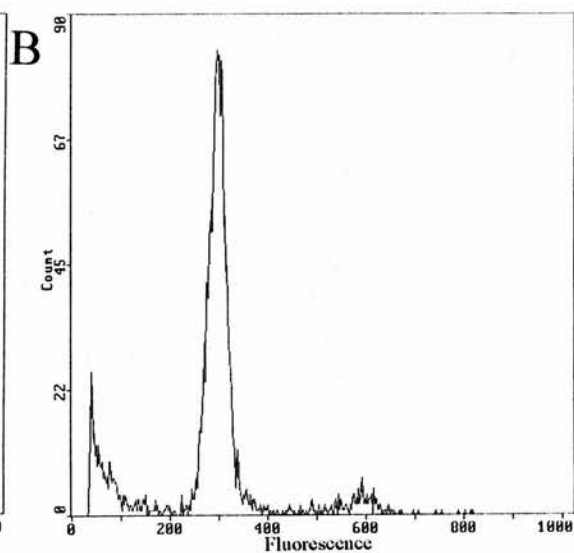
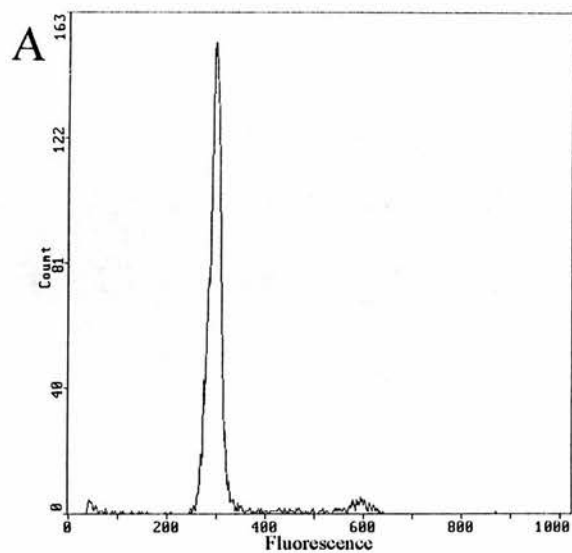


## **Figure 12**

### **Flow cytometric analysis of sporadic colorectal cancers xenografts**

Flow cytometry of tumour cells from the following sources:

- (A) Primary tumour corresponding to xenograft SAGR,
- (B) 1st passage xenograft SAGR, showing large near-diploid peak,
- (C) Primary tumour corresponding to xenograft HIDE,
- (D) 3rd passage xenograft HIDE with large aneuploid peak, and
- (E) normal colonic tissue.



### 5.3.4 Assessment of chromosomal changes by Comparative Genomic Hybridisation

#### *5.3.4.1 Chromosomal copy number changes detected by CGH*

DNA copy number changes were assessed by CGH in xenografted tumours derived from 28 primary colorectal tumours and one lymph node metastasis. Examples of CGH hybridisations and ratio profiles are shown in Figure 13, and a summary of the chromosomal anomalies detected in all tumours is presented in Figures 14 and 16. The vast majority of abnormalities were deletions or amplifications of either whole chromosomes or entire chromosome arms. Duplications and deletions which involved interstitial breakpoints are presented in Figure 15. The most frequently observed changes, occurring in over half of all tumours, were deletion of chromosomes 8p and 18q and duplication of chromosomes 8q, 13q and 20q (Figures 14 & 16). These chromosomal abnormalities were notable in that they could often still be observed when the ratio cut-off point was increased to the equivalent of loss or gain of one chromosome in 75% diploid cells, indicating their presence as the majority clonal population. Also very frequent were duplications of chromosome 7q and 7p and deletions of 17p, occurring in around half the series. Deletions of 1p, 5q, 18p and 15q were also common (Figures 14, 15 & 16). Sex chromosome anomalies were also fairly frequent, with loss of X in 7 tumours and loss of Yp in 7/28 tumours (25%). However, because males were assumed to be less likely to lose their single X chromosome than females were to lose one copy (supported by the fact that all 7 tumours with X chromosome loss were from female patients), sex chromosomes were omitted from statistical analysis.

Kallioniemi *et al.* (1994) suggest that some chromosomal regions, namely 1p32-pter, 16p, 19 and 22, can give aberrant results, probably because of their high guanine/cytosine content. In this study, normal versus normal controls, which were included in each experiment, usually demonstrated even hybridisation of DNA to all chromosomes, although chromosome 19 occasionally gave aberrant results. Reversal of the usual red to green labelling of tumour and normal DNA demonstrated chromosome 19 to be the only region of inconsistency. Results from this chromosome are included but should be interpreted cautiously.

**Figure 13**

**Example of Comparative Genomic Hybridisation.**

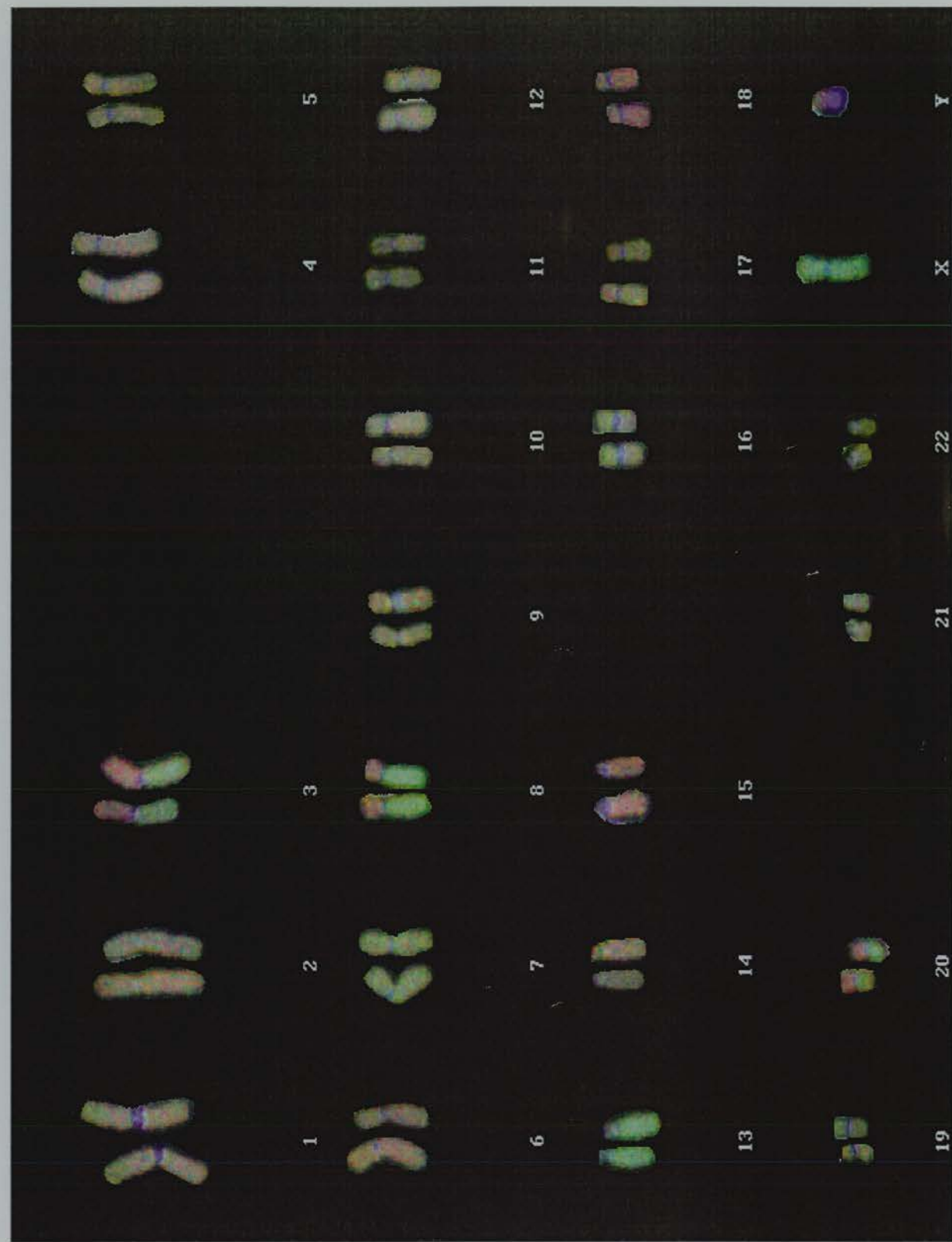
Comparative Genomic Hybridisation of one xenografted tumour, CADU, and normal control:

- (A) Raw, unaltered metaphase image, CADU.
- (B) The same metaphase after karyotyping and background subtraction.
- (C) Ratio profile of an average of 6 CADU metaphases.
- (D) Ratio profile of an average of 6 metaphases from a normal vs normal control.

***a***

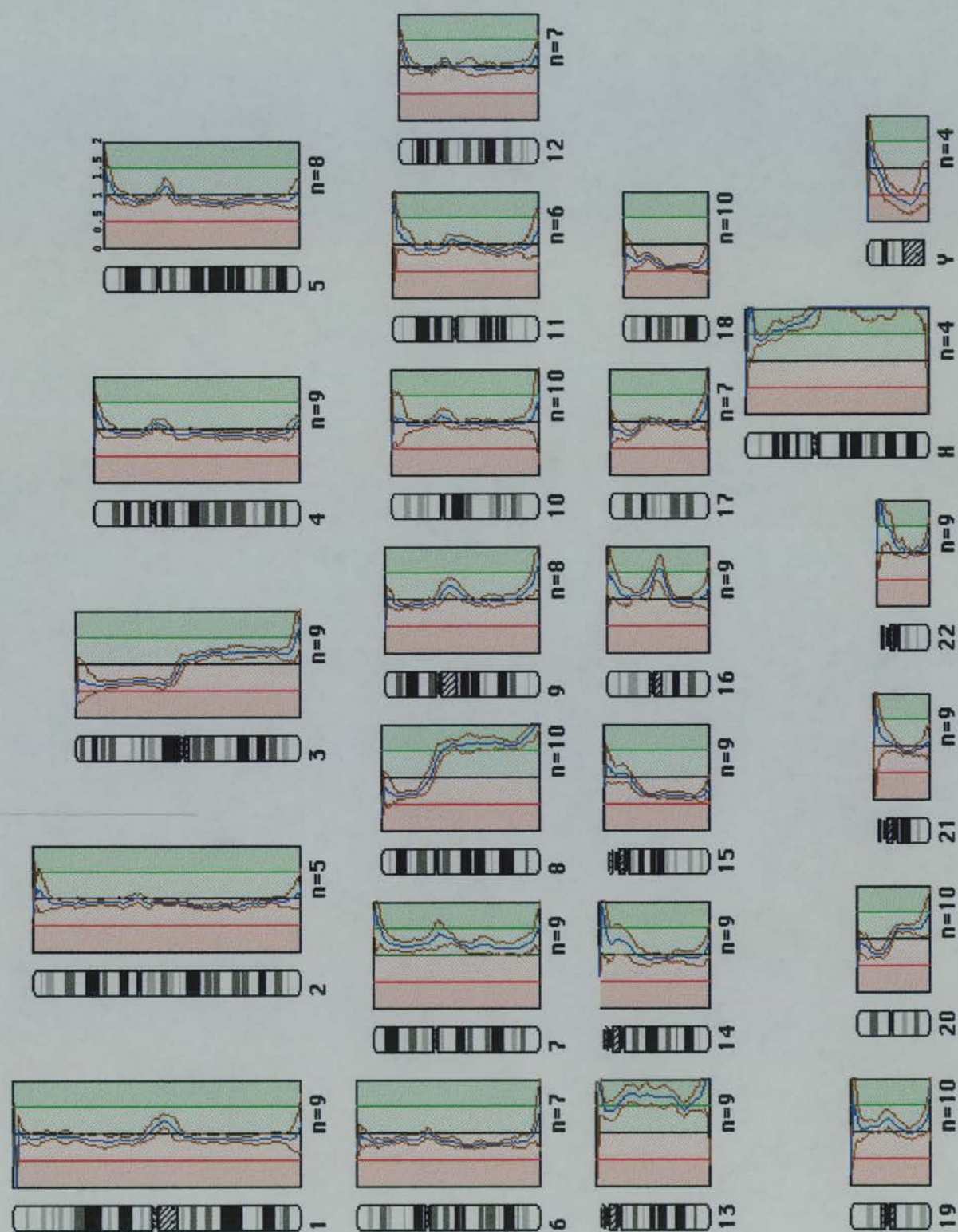


***b***

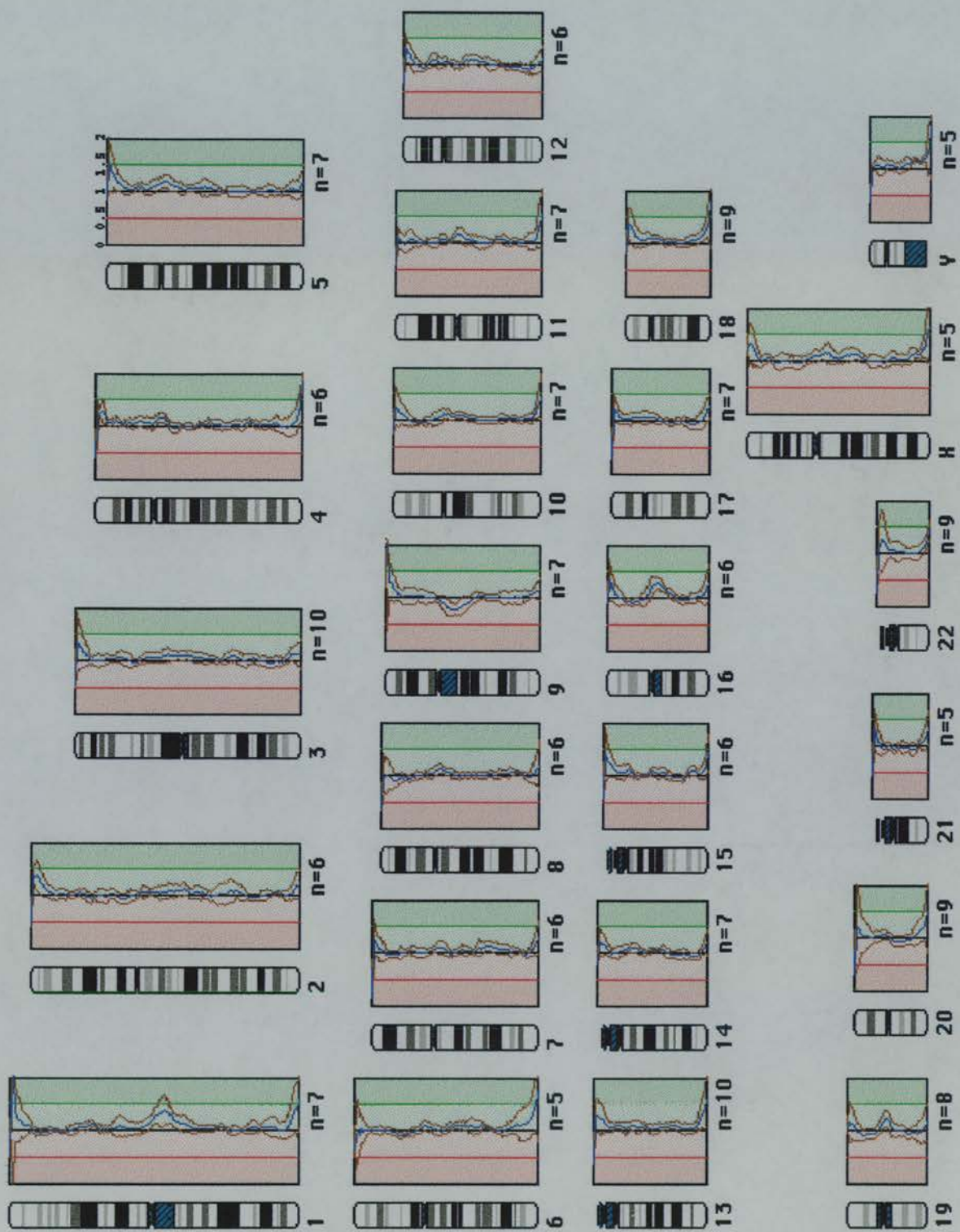




C



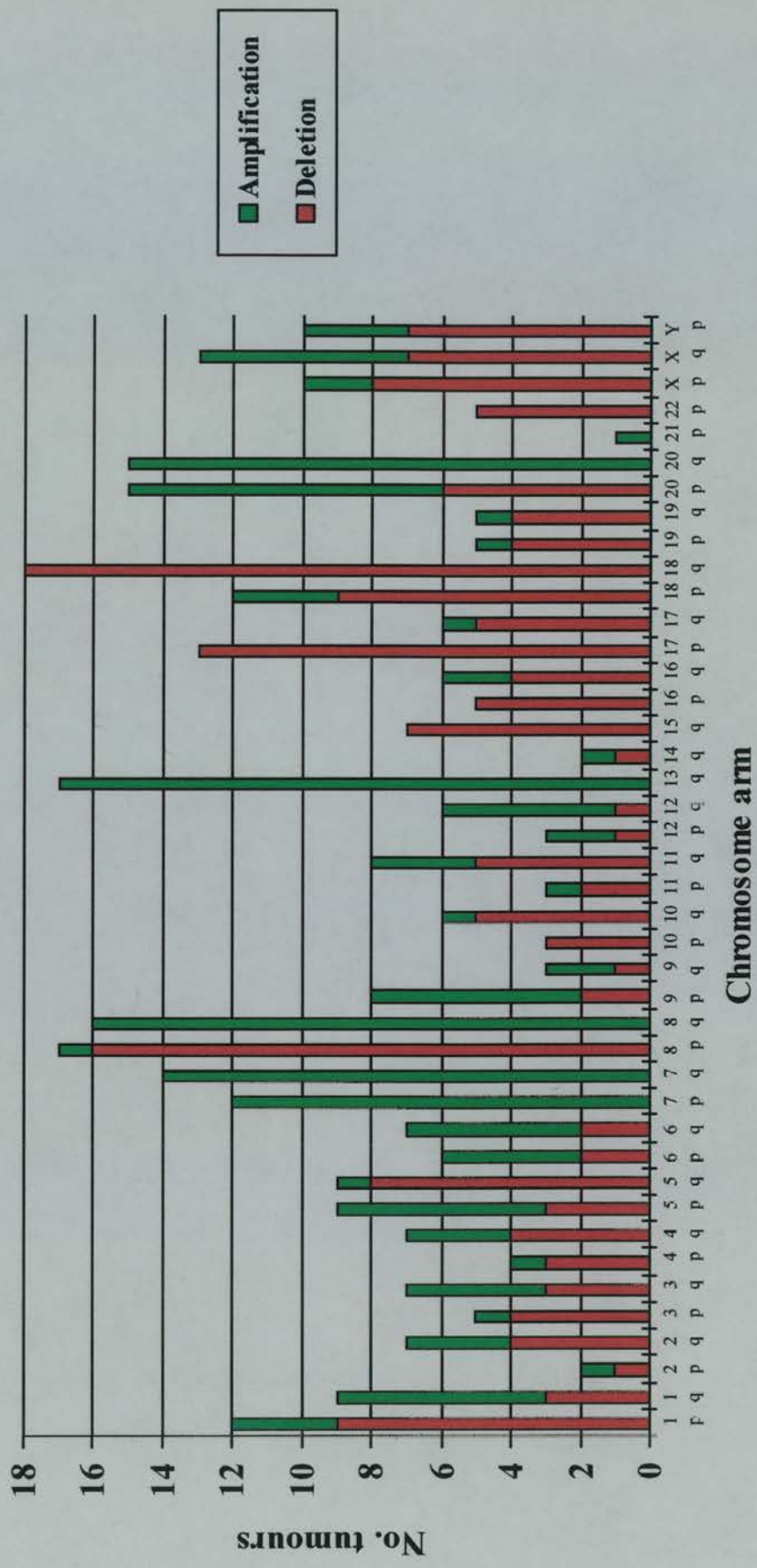




## **Figure 14**

### **Summary of chromosomal abnormalities in sporadic colorectal cancer xenografts**

This graph illustrates the number of deletion (red) and amplification (green) events occurring on each chromosome arm. These events always involved the entire chromosome arm unless shown as an interstitial deletion/amplification in Figure 15.

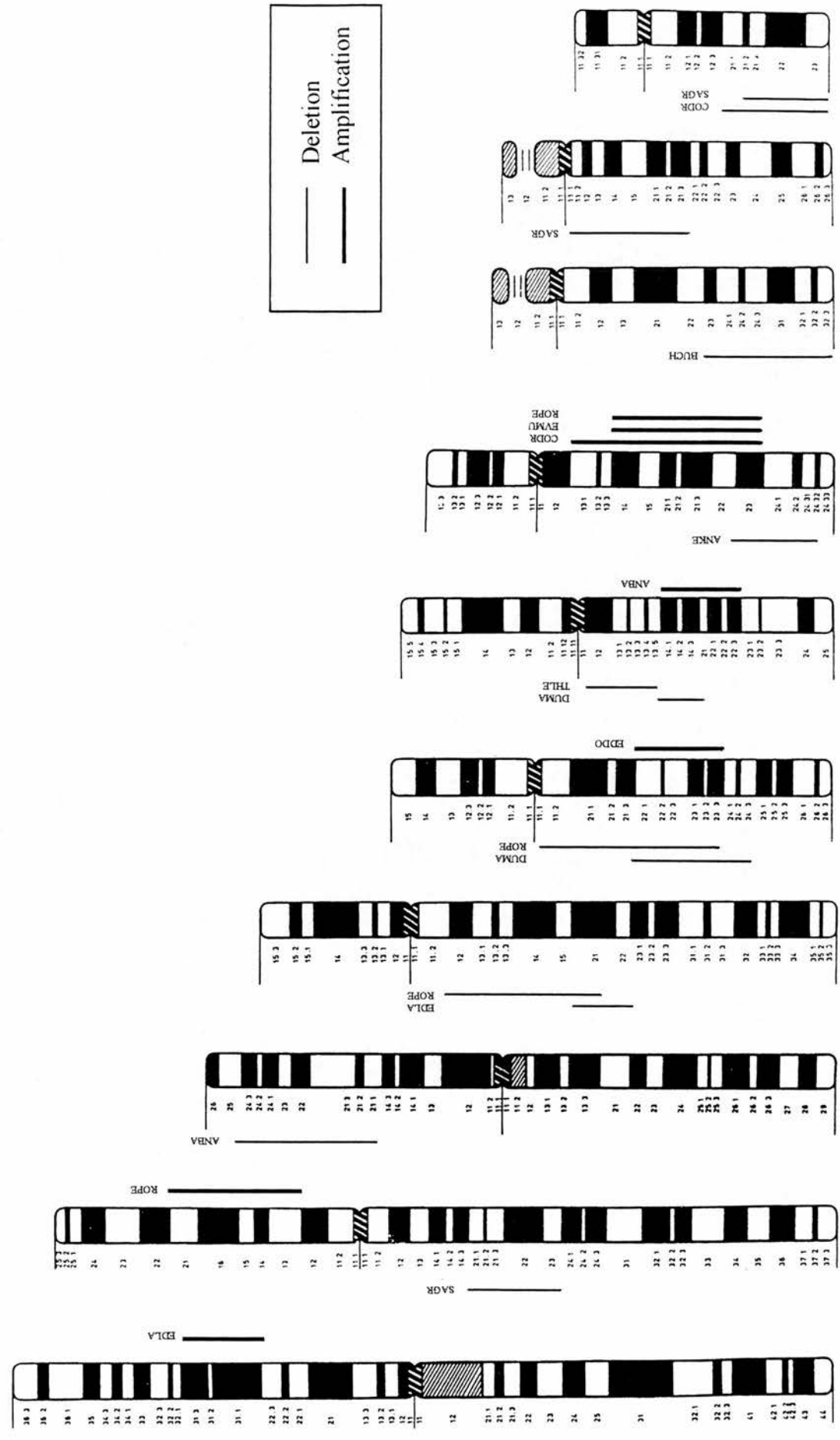


**Figure 15**

**Discrete deletions and amplifications in colorectal cancer xenografts**

Amplifications and deletions necessarily involving non-centromeric interstitial breakage occurred in twelve tumours, and are shown here. Deletions are shown to the left of each chromosome with a narrow line. Amplifications are shown to the right with a wide line.

HIDE  
EVMTU  
SAGR  
ROPE  
EDDO



1 2 3 5 10 11 12 14 15 18

#### **5.3.4.2 Chromosome abnormalities assessed by CGH in relation to DNA ploidy**

Chromosomal copy number changes assessed by CGH are relative to the total DNA content of the sample and do not take into account overall DNA content. Tumours which had been assessed as aneuploid or near-diploid by flow cytometry were compared to determine whether numbers of relative copy number changes within them, detected by CGH, differed. When the number of chromosomal losses in diploid and aneuploid tumours was compared, no significant difference was found ( $p=0.7499$ , Mann-Whitney test). However, significantly fewer chromosomal gains occurred in near-diploid tumours ( $p=0.0008$ , Mann-Whitney test).

#### **5.3.4.3 Chromosome abnormalities and microsatellite instability status**

Four of 5 RER+ cancers available for assessment by flow cytometry were near-diploid, compared with only 3/22 RER- tumours ( $p=0.016$ , 2-tailed Fisher's exact test). One RER+ cancer apparently underwent endoreduplication between the primary tumour and time of harvesting as xenograft, and was excluded from statistical analysis.

When numbers of chromosomal changes detected by CGH in RER+ cancers were compared with RER-, RER+ lesions were significantly less likely to incur chromosomal gains ( $p=0.0426$ , Mann-Whitney test). The total number of changes occurring per tumour was not significantly different in RER+ tumours, nor were the total number of losses ( $p=0.1035$  and  $p=0.1959$ , respectively, Mann-Whitney test).

A striking pattern of chromosomal abnormalities was observed in RER- cancers compared with RER+. At least two of the frequent chromosomal abnormalities, deletion of chromosome 8p and 18q, and duplication of 8q, 13q and 20q, were present in 21/22 RER- cancers. Only one of the six RER+ cancers had more than one of these abnormalities, despite displaying changes in other chromosomes (although three other RER+ tumours had one of these abnormalities each), a difference which was highly significant ( $p=0.0004$ , Fisher's exact test). RER+ cancers showed no loss of chromosome 8p or 18q at all, compared with one or both changes in 20/22 RER- cancers ( $p<0.0001$ , Fisher's exact test), despite the overall levels of loss being comparable.

#### **5.3.4.4 Chromosome abnormalities and p53 status**

Classification of p53 status proved complicated. Flow cytometric analysis revealed no significant difference in DNA ploidy between the group of 6 tumours (HIDE, WITO, GRZBO, JEDO, EVMU and MEMCG) in which p53 was apparently normal on the grounds of negative IHC and absence of mutation and those in which p53 was mutated or stabilised; 2/5 tumours in which p53 was normal by IHC and mutation analysis were near-diploid, compared with 5/19 where p53 was abnormal ( $p=0.642$ , 2-tailed Fisher's exact test). Similarly, there was no significant difference between occurrence of chromosome loss or gain



detected by CGH in tumours with defective p53, defined by the same criteria, and those in which p53 was apparently normal.

However, CGH analysis revealed clear loss of 17p, the chromosomal region containing p53, in three cancers which had been classified by IHC and mutation analysis as normal for p53. Loss of 17p is strongly correlated with mutations of the remaining allele of the p53 gene and abnormalities of protein function (Baker *et al.*, 1990; Cunningham *et al.*, 1992). When these three tumours were reclassified as defective for p53, only three remained which could be classified as 'p53 normal', GRZBO, JEDO and MEMCG. These three tumours had very few chromosomal changes, and when the test was repeated including only these three cancers as 'p53 normal', a significantly higher number of chromosomal gains and losses were seen in p53 defective tumours compared with p53 normal ( $p=0.0399$  and  $p=0.0153$ , Mann-Whitney test). Moreover, a further two tumours in this series (JEGA and SAGR) displayed very low levels of immunohistochemical staining for p53, where the percentage of nuclei staining positive with DO7 was 1% or less, and had no detectable mutation of the p53 gene. Low levels of immunohistochemical staining of p53 correspond very infrequently with mutation of the p53 gene (Cripps *et al.*, 1992), and therefore the positive nuclear staining in these tumours may represent high levels of wild-type protein (discussed in Wynford-Thomas, 1992; Hall & Lane, 1994). The test was repeated including as 'p53 normal' tumours with low levels of IHC staining as well as those with no evidence of mutation or loss of 17p. The number of chromosomal gains and losses were significantly lower in these tumours compared to those with higher levels of staining or mutation of p53 ( $p=0.0019$  and  $p=0.0055$ , Mann-Whitney test).

No pattern of chromosomal abnormality was associated with either p53 status; all of the common anomalies described in 5.3.4.1 occurred in both p53 defective and p53 normal cancers.

In this series, loss of chromosome 17p did not correlate with detected positive immunocytochemical p53 status as has been demonstrated in another series of colorectal cancers (Cunningham *et al.*, 1992). Loss of 17p occurred in 9/20 IHC positive lesions and 4/8 IHC-negative tumours. Although not all tumours were assessed for p53 mutation, only 2/6 with mutation had detectable loss of 17p material. However, losses of this region were not examined by methods more sensitive than CGH.

#### 5.3.4.5 *Chromosome abnormalities and clinicopathological features*

The numbers of chromosomal losses and gains were examined in relation to the side of the bowel in which the primary cancer was located, and to Dukes' stage. There was no difference between the number of chromosomal changes in left-sided compared with right-



sided cancers, or in Dukes' A & B compared with Dukes' C & D cancers. Furthermore, no pattern of chromosomal change predominated in any of these groups.

#### ***5.3.4.6 Chromosome abnormalities in a lymph node metastasis***

CGH analysis of the tumour derived from a lymph node metastasis showed a high number of chromosomal changes, including two of the common alterations, gain of 20q and 8q. However, this cancer did not demonstrate loss of 18q, an event suggested by others to occur ubiquitously in metastatic colorectal cancer (Ookawa *et al.*, 1993; Frank *et al.*, 1997).

#### ***5.3.4.7 Comparison of xenografted and primary tumour***

In order to confirm that chromosomal abnormalities in xenografted tumours were comparable with those of the original primary tumour, CGH was carried out on three primary tumours from which early passage xenografts were derived. In one case (WITO), the primary tumour was essentially identical to the early passage xenograft, except for a more marked loss of chromosome 20p in the xenograft. Xenograft CADU represented all the main changes present in the primary tumour (loss of 8p, 15q, 17p and 18 and gain of 8q, 13q and 20q), but appeared to have undergone three further events: pronounced loss of 3p, duplication of 3q and loss of 20p. Xenograft JOCR appeared to represent a subclone of the original tumour, showing more marked appearance of certain chromosome abnormalities evident in the primary tumour; conversely, certain other changes that were prominent in the primary tumour (gain of 12p and some of 12q, gain of 18p and loss of 22q) were present at lower levels in the xenograft. Interestingly, striking loss of 8p was present in both primary tumour and xenograft, indicating that this event must have preceded the divergence represented by the differences between primary tumour and xenograft. Three lesions unequivocally present in the primary tumour corresponding to xenograft JOCR could not be detected at all in the xenograft: 18q loss, 20q gain and Yp loss.

**Figure 16**

**Chromosomal abnormalities in xenografted sporadic colorectal cancers according to RER and p53 status.**

Chromosome deletions (red) and amplifications (green) are shown for each xenografted colorectal tumour from the series of 28. Each horizontal row represents a tumour, each vertical column a chromosome arm. A chromosome arm was scored red if it all or part of it reached the threshold for deletion (0.875), as green if all or part of it reached the threshold for amplification (1.125), or as red and green if both amplification and deletion occurred.

RER status is given as positive (+) or negative (-). p53 status is given as follows: defective by either positive IHC at >1% nuclei and/or mutation analysis (D); defective only by positive IHC at ≤1% of nuclei, but with no detectable mutation (D\*); normal by IHC and mutation analysis and showing no deletion of 17p material (N); normal by IHC and mutation analysis but showing loss of chromosome 17p by CGH (N\*). The xenograft derived from a lymph node metastasis (LN), ANSA, is shown at the bottom but was not included in statistical analysis.

Turnour name	RER status	p53 status	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	X	Y
			p	q	p	q	p	q	p	q	p	q	p	q	p	q	q	p	q	p	q	p	q	p	q	p	q
ANBA	-	D																									
ANWO	-	D																									
BUCH	-	D																									
CADU	-	D																									
CORU	-	D																									
DUMA	-	D																									
EDDO	-	D																									
EDLA	-	D																									
ELWI	-	D																									
GEHA	-	D																									
JAMU2	-	D																									
JOCR	-	D																									
JOLO	-	D																									
PAMCI	-	D																									
ROPE	-	D																									
SAGR	-	D*																									
THLE	-	D																									
CODR	-	D																									
HIDE	-	N*																									
WITO	-	N*																									
GRZBO	-	N																									
JEDO	-	N																									
EVMU	+	N*																									
MEMCG	+	N																									
AGSC	+	D																									
ANKE	+	D																									
MACA	+	D																									
JEGA	+	D*																									
ANSA(LN)	-	D																									

## 5.4 Discussion

Genomic instability is intrinsic to carcinoma development, and numerous types of instability have been demonstrated in colorectal neoplasms. The present study has used CGH to examine the complex chromosomal abnormalities which have evolved in a series of such tumours in relation to putative mechanisms of instability: defects of p53 and of mismatch repair, inferred by the presence of microsatellite instability.

The chromosomal deletions and amplifications found here conform with many other cytogenetic and CGH studies of colorectal carcinomas (Reichmann *et al.*, 1981; Muleris *et al.* 1988, 1990; Yaseen *et al.*, 1990; Konstantinova *et al.*, 1991; Bardi *et al.*, 1993b; Barletta *et al.*, 1993; Herbergs *et al.*, 1994; Gerdes *et al.*, 1995; Schlegel *et al.*, 1995; Ried *et al.*, 1996). In addition, others have shown that RER+ colorectal cancers tend to remain near-diploid (Aaltonen *et al.*, 1993; Lothe *et al.*, 1993) and the RER+ cancers in this series conform with this finding (although one case, ANKE, was unusual and is discussed below). However, whilst global DNA ploidy generally remains near-normal in RER+ tumours, the CGH analysis presented here demonstrates that chromosomal changes do occur: the number of chromosomal deletion events in RER+ tumours are similar to those of the RER- group, although significantly lower numbers of individual chromosome duplications are present in RER+ tumours. Thus, it appears that a mechanism by which chromosomal stability is maintained may be deficient in at least some RER+ tumours. A similar CGH study found smaller numbers of chromosomal abnormalities, especially duplications, in RER+ colorectal cancers compared with RER- (Schlegel *et al.*, 1995). Although numbers in the study by Schlegel *et al.* were small and direct comparison is difficult because the authors do not give details of their threshold for scoring chromosomal changes, these data appear to be in keeping with this study. Somewhat at odds with these results, cytogenetic analysis of colorectal cancers found the RER+ phenotype to be associated with either normal or 'trisomic type' karyotype, in which duplications are common but structural rearrangements rare (Remvikos *et al.*, 1995). This discrepancy is difficult to explain, but may partly be accounted for by the small numbers involved in each study and the inability to distinguish structural rearrangements, and thus 'karyotypic type', by CGH (Muleris *et al.*, 1990). Despite some differences, taken together, these studies suggest that the number of chromosomal abnormalities is lower, though by no means absent, in RER+ colorectal cancers. There is some evidence that defective mismatch repair itself could contribute to chromosomal instability (de Wind *et al.*, 1995; Baker *et al.*, 1995 & 1996; Reitmair *et al.*, 1995; Edelmann *et al.*, 1996), but compelling *in vitro* data shows that chromosomal instability exists as a distinct phenomenon which can coexist with microsatellite instability in colorectal cancer cells (Lengauer *et al.*, 1997b).



Whilst mechanisms driving some level of chromosomal instability appear to be active in RER+ cancers, a remarkable 95% of RER- tumours from this series demonstrated a distinctive pattern of deletion and amplification rarely observed in RER+ tumours. These abnormalities involved a combination of at least two of the following changes: deletion of 8p and 18q and duplication of 8q, 13q and 20q. More striking still, 8p and 18q deletion, at least one of which was present in 91% of RER- cancers, was not observed in any of the RER+ tumours. These patterns provide a tantalising insight into the underlying mechanisms of instability and selection in colorectal cancer. The pattern of chromosomal abnormalities observed in RER- cancers is probably the result of selective pressure in favour of cells in which such anomalies have disrupted genes critical to tumorigenic development. These clonal abnormalities do not arise in RER+ cancers, and this could be because any chromosomal instability present does not predispose to such changes, or because clonal selection for them is not favoured. Clonal selection for these particular chromosomal abnormalities might not be favoured if frameshift and point mutations occur so frequently that they invariably cause mutations within critical genes, making the RER+ phenotype effectively dominant. A current hypothesis suggests that a similar, though probably not identical, combination of oncogenes and tumour suppressor genes are important to the development of all colorectal cancers, but the way in which these genes are mutated differs, occurring predominantly through frameshift and point mutations in RER+ and through larger chromosomal events in RER- (Kinzler & Vogelstein, 1996). It is not possible to discern from these results why this pattern of chromosomal loss and gain is not observed in RER+ tumours, but mutation analysis in RER+ tumours of the putative tumour suppressor genes located in these regions may help to explain. The tumour suppressor genes DCC (Vogelstein *et al.*, 1988; Fearon *et al.*, 1990; Itoh *et al.*, 1993; Cho *et al.*, 1994) DPC4 (Takagi *et al.*, 1996; Thiagalingam *et al.*, 1996) and J18 (Riggins *et al.*, 1996) are all located on the frequently lost chromosome 18q, whilst the frequent deletion of chromosome 8p supports evidence from molecular studies for the presence of at least one, and probably two, tumour suppressor genes in this region (Cunningham *et al.*, 1993; Fujiwara *et al.*, 1993; Yaremko *et al.*, 1994; Farrington *et al.*, 1996). The significance of duplication of chromosomes 8q, 13q and 20q to the cancer phenotype is not clear, since the regions of amplification include such large portions of the genome in which no genes of known importance in colorectal cancer are located.

How do chromosomal anomalies arise? Although numerical and structural chromosomal anomalies do not necessarily arise through similar mechanisms, abnormality of a single gene product with multiple functions in chromosome stability regulation, such as p53, could allow both types of instability to arise simultaneously. Structural chromosomal instability can be initiated by a small number of events (such as strand breakage or telomere loss), resulting in activation of the breakage-fusion-bridge (BFB) mechanism, in which sister

chromatid- or aberrant chromosomal fusion is followed by a sequence comprising repeated breakage and fusion which eventually leads to stable, though abnormal, chromosomes (McClintock, 1951). Generation of stable chromosomes may occur if breakage events have occurred within intrachromosomal telomere-like repeats or centromeric sequences, when such sequences are brought to the end of chromosomes. If preferential breakpoints are present within the chromosomes in which chromosome arms often show loss or gain, especially chromosome 8, these could facilitate the acquisition of anomalies involving these chromosomes through frequent generation of stable abnormal structures. Removal of a DNA damage surveillance component could allow accumulation of the type of damage capable of initiating this cycle, as well as propagation of cells in which DNA damaged by the BFB cycle is allowed to replicate.

Could p53 defects account for the generation of chromosomal anomalies? Very few chromosomal changes were seen in three tumours where no p53 defect could be detected by immunohistochemistry and mutation analysis and where loss of 17p was not visible. Similarly, tumours in which very low levels of IHC staining were present, where positive staining may be due to over-expression of wild-type protein in response to elevated levels of DNA damage (Maltzman *et al.*, 1984; Barnes *et al.*, 1992; Vojstek & Lane, 1993), and no mutation or loss of the gene occurred, had very low levels of chromosomal abnormality. Both of these categories are likely to define cancers in which there is no underlying defect of p53, and so these data probably suggest, in keeping with many other studies (Bischoff *et al.*, 1990; Livingstone *et al.*, 1992; Yin *et al.*, 1992; Carder *et al.*, 1993 & 1995; Agapova *et al.*, 1996; Remvikos *et al.*, 1997), that wild-type p53 activity is associated with low levels of chromosomal abnormality. However, finding an appropriate definition of normal and abnormal p53 status clearly presents problems. Lesions with no IHC staining for p53 could harbour p53 abnormalities which render the p53 protein undetectable by immunocytochemical methods by the formation of epitopes not detectable by either antibody DO7 or pAb1801, through truncation (Remvikos *et al.*, 1997) or altered conformation (Cripps *et al.*, 1992) of the protein. Conversely, stabilisation of the protein is not absolutely correlated with missense mutation (Gannon *et al.*, 1990; Cunningham *et al.*, 1992; Cripps *et al.*, 1992; Baas *et al.*, 1994), and several factors other than gene mutation, including binding to viral proteins (Lane *et al.*, 1990; Levine *et al.*, 1991) and to cellular factors such as *Mdm2* (Wu *et al.*, 1993) can lead to inefficient protein breakdown and stabilisation of the protein. In addition, selection against deletion of 17p in tumours classified as normal for p53 introduces bias, since this involves some selection against the process of chromosomal instability in its own right. However p53 status is defined, the association between anomalies of p53 and presence of chromosomal defects is not absolute; it is possible for few chromosomal changes to be present in conjunction with abnormal p53 stabilisation, and chromosomal anomalies occur to some

degree in tumours without p53 deficiency, though the methods used do not rule out mutations elsewhere in the gene.

Most chromosomal abnormalities detected in this study involved either whole chromosome loss/gain or apparent centromeric breakage. One exception was deletion of chromosome 1p, in which recurrent loss occurred in patterns consistent with the presence of at least two tumour suppressor genes (Figure 15). This conforms with data from more detailed loss of heterozygosity studies, in which 1p has been suggested to harbour at least 3 tumour suppressor genes involved in colorectal cancer development (Leister *et al.*, 1990; Bardi *et al.*, 1993a; Praml *et al.*, 1995a). Smaller interstitial losses, such as deletions within chromosome 5q, may be under-represented because slight errors in measurement of signal position along the chromosome, caused by small differences in chromatin condensation between metaphases, can lead to damping down of small, sharp peaks in the fluorescence ratio. In the case of chromosome 5q, interstitial deletions which reached the chosen ratio cut-off level were present in only two tumours, whereas four others showed evidence of loss not reaching this level in their fluorescence ratios. Data from this laboratory indicate that a high frequency of relatively small deletions do indeed occur in this region (Ashton-Rickardt *et al.*, 1989 & 1991; Curtis *et al.*, 1994). Software under development employs band-by-band chromosome measurement, rather than the current end-to-end method, and should provide more accurate analysis. Small discrete amplifications were extremely rare, indicating that, unlike other tumour types such as cancer of the breast (Muleris *et al.*, 1994b), discrete gene amplification probably does not represent an important mechanism of oncogene activation in colorectal tumorigenesis.

The investigation has provided further evidence of the value of xenografted tumour material as a study model representative of the progenitor primary tumour. Although the comparison of three primary tumours with corresponding xenografts presented here clearly demonstrates that some degree of chromosomal evolution can occur during growth, as would be expected if the underlying process of chromosomal instability is a dynamic one (Lengauer *et al.*, 1997b), the major chromosomal lesions present in the primary tumour are usually retained. Complete concordance between primary tumour and xenograft was observed with p53 immunohistochemistry, and only one discrepancy occurred with the results of flow cytometry. In this case, tumour ANKE appeared to have undergone endoreduplication, changing from a diploid state in the primary tumour to tetraploid in the xenograft. Xenografted colorectal tumours have been suggested to be somewhat more prone to endoreduplication than primary tumours (Lefrançois *et al.*, 1989), but the close adherence of the chromosomal anomalies detected here to the patterns seen in many larger studies suggests that xenografted tumours do not undergo significant changes as a consequence of transposition into the mouse environment. In addition, preliminary data from six late passage xenografts suggests that very little chromosomal evolution occurs between early and late pass



tumours. The fact that this study includes cancers of all Dukes stage, tumour site, tumour type, histological differentiation, ploidy level, p53 status and RER status supports data indicating that tumorigenicity in nude mice is independent of many, though not all, prognostic factors (Jessup *et al.*, 1988; Lefrançois *et al.*, 1989).

Despite retaining characteristics of the primary tumour, subcutaneous xenografts undergo infrequent invasion and metastasis (McQueen *et al.*, 1991). However, injection of colon cancer cells into the caecal wall of athymic nude mice results in lymphatic and vascular invasion and a pattern of metastasis similar to that seen in human colonic cancer (Bresalier *et al.*, 1987a & b), suggesting the involvement of tissue-specific factors in the modulation of tumour development. Investigation of alternative methods of implantation may lead to the development of a tumour model still more representative of primary colorectal cancer than the subcutaneous grafts used here.

## 5.5 Conclusions

In summary, this study has addressed the relationship between chromosomal abnormalities and two putative mechanisms by which tumorigenesis is driven, namely replication error defects and abnormalities of p53. Clear differences between chromosomal abnormalities in RER+ and RER- cancers have emerged, and, though there appears to be an association between abnormal p53 status and high levels of chromosomal abnormalities, difficulties of assigning p53 status mean that this cannot be said with certainty. In addition, further confirmation of the value of xenografts as good study models for colorectal cancer has been provided. The need for analysis of larger numbers of tumours is evident, and this investigation provides the basis for a larger study of colorectal tumours, currently underway, in which it is hoped the patterns of chromosomal change demonstrated here will be confirmed.

## CHAPTER 6

### GENETIC INSTABILITY IN MURINE TUMOURS DEFICIENT IN p53 AND MSH2

#### 6.1. Introduction

Defects of p53 and of the mismatch repair pathway occur frequently in human colorectal cancer, and are thought to be fundamental to tumour progression. The generation by gene targeting of mice with defects of both p53 and components of mismatch repair provides a relatively uncomplicated system in which to study the contribution of each factor to development and tumorigenesis. Mice in which p53 is homozygously defective are developmentally normal but succumb to tumours, predominantly lymphomas, by the age of 9-10 months (Donehower *et al.*, 1992; Harvey *et al.*, 1993a; Purdie *et al.*, 1994). *In vitro*, cells derived from such mice have very unstable karyotypes, with a strong tendency to increase their ploidy levels during growth (Harvey *et al.*, 1993b; Purdie *et al.*, 1994) and an ability to undergo gene amplification (Livingstone *et al.*, 1992; Yin *et al.*, 1992). Heterozygous mice acquire a somewhat different spectrum of tumours, predominantly lymphomas and sarcomas, at a later age (Harvey *et al.*, 1993a; Purdie *et al.*, 1994). Mice with homozygous deletion of *Msh2* are also healthy at birth, but around one third develop tumours, mainly metastasizing lymphomas of T-cell origin, in the first year (de Wind *et al.*, 1995; Reitmair *et al.*, 1995). Development of intestinal tumours is common in older mice with lymphoma, and skin lesions are sometimes found (Reitmair *et al.*, 1996a). Both tumour tissue and non-tumorigenic cells from *Msh2* null mice (though not normal tissue *in vivo*) frequently display widespread microsatellite instability in culture, and cells have been shown to lose heterology-dependent suppression of recombination *in vitro*. Mice with heterozygous loss of *Msh2* have functional mismatch binding and do not develop any more tumours than wild-type controls (Reitmair *et al.*, 1995 & 1996a). Thus, the mutational events occurring in cells from such mice are largely consistent with those of human cells with comparable genotype, where p53-deficiency apparently predisposes to gross chromosomal abnormalities whilst lack of hMSH2 favours acquisition of point mutations and microsatellite instability.

Interbreeding of mice deficient in genes of importance in tumour development could prove invaluable to our understanding of how genetic events contribute to cancer initiation and progression. *Msh2*-deficiency has been shown to accelerate the rate of adenoma formation in *Apc*<sup>+/-</sup> mice, apparently through point mutation of the remaining *Apc* allele (Reitmair *et al.*, 1996b). Interbreeding of mice deficient in both *Msh2* and *p53* also accelerates tumorigenesis, suggesting the two defects to be co-operative for tumorigenesis (Cranston *et al.*, 1997; Toft

*et al.*, 1998). One report demonstrates female embryonic lethality for this genotype, with female embryos exhibiting massive levels of global apoptosis (Cranston *et al.*, 1997), though liveborn females were present in animals bred in our laboratory (Toft *et al.*, 1998). Tumour tissue from mice deficient in both p53 and Msh2 function displays microsatellite instability at a similar rate to that in *Msh2*-deficient tumours alone (Cranston *et al.*, 1997), but chromosomal instability in such lesions has not yet been studied.

This chapter extends the analysis of the p53- and mismatch repair-deficient phenotype presented in Chapter 5 by examining microsatellite instability and chromosomal abnormalities in mice with known deficiencies of both p53 and Msh2. The examination of these traits in human tumours can be problematic, and analysing these defects in murine systems avoids numerous difficulties, notably those associated with the intervention of many other genetic events in the cancer phenotype and the considerable difficulties of accurate classification of p53 status (discussed in Chapter 5). In addition to mice with null genotypes for both genes, study of the effects of heterozygosity at either locus was made possible by the availability of tumour tissue from animals with heterozygosity at one locus and homozygosity at the other. The aim, in keeping with that of the previous chapter, was to examine the interaction of the two tumorigenic pathways and to determine which pathway, if any, produces the predominant pattern of genomic instability.

## 6.2. Materials and methods

Unless otherwise stated, the methods employed in this chapter are described in 2.14. *Msh2*<sup>-/-</sup> mice were interbred with *p53*<sup>-/-</sup> mice to generate F2 animals mutant for both genes. Genotype was confirmed using a PCR-based assay. Following DNA extraction from lymphomas arising in these animals, CGH was carried out on a series of 42 tumours using a protocol almost identical to that employed with human DNA, using a similar software package but with manual karyotyping. Hybridisation quality was verified by the correct identification of sex according to X chromosome copy number. A green/red (tumour/normal) ratio cut-off point of 1.2 was chosen for delineation of amplifications and 0.8 for deletions, representing gain or loss of chromosomal material in 40% of cells. This ratio, somewhat higher than that chosen for CGH analysis of human chromosomes, was chosen in order to be certain of scoring only genuine aneuploidy; intrinsic properties of the software occasionally resulted in generalised skewing of the euchromatin ratio away from the ratio=1 line due to extreme green/red ratios in the heterochromatic regions, which are more abundant in the mouse than in the human genome. This skewing, though uniform, resulted in difficulties of interpretation, particularly if aneuploidy was apparent. Additionally, no tumours of known karyotype were available as positive controls, and karyotypes have not previously been published for tumours arising in these mice, so a higher ratio cut-off point was felt to be safer. Consequently, chromosomal changes may be underestimated.

Matched normal tissues were not available for many of the tumours analysed for CGH. Analysis of microsatellite sequences was carried out on 20 matched normal/tumour pairs, using four polymorphic microsatellite loci, D1Mit4, D7Mit17, D10Mit2 and D14Mit15, as in 2.14.3. 8 of these tumours were assessed by CGH.

Flow cytometry was performed on a set of 28 tumours not included in the CGH study, using the method of Vindelov (1993) as described in 2.5.1. In addition, loss of heterozygosity analysis of *p53* and *Msh2* was undertaken using same the PCR-based assays that were used for genotyping (2.14), and DNA sequencing was carried out on three tumours using a dideoxy chain termination method (2.9.2).

### 6.3. Results

#### 6.3.1. Tumour development in knockout mice

The median age at which mice null for *Msh2* alone (*Msh2*<sup>-/-</sup> *p53*<sup>+/+</sup>) died or were killed because of tumour development was 142 days. For *p53* null animals (*Msh2*<sup>+/+</sup> *p53*<sup>-/-</sup>) it was significantly earlier, at 121 days ( $p < 0.0006$ , log rank test). Mice totally deficient for both *p53* and *Msh2* (*Msh2*<sup>-/-</sup> *p53*<sup>-/-</sup>) died at 65 days, earlier than those null for either defect ( $p < 0.0006$ , log rank test), indicating that *p53* and *Msh2* are co-operative for tumorigenesis.

Animals heterozygous for *p53* deficiency but with homozygous loss of *Msh2* function (*Msh2*<sup>-/-</sup> *p53*<sup>+/-</sup>) had a median age of death of 99 days, earlier than homozygous deficiency of *Msh2* alone ( $p < 0.00001$ , log rank test) whilst those totally lacking in *p53* and heterozygous for *Msh2* (*Msh2*<sup>+/-</sup> *p53*<sup>-/-</sup>) died at 109 days, which was not significantly earlier than *p53*<sup>-/-</sup> mice ( $p > 0.09$ , log rank test). Figures were based on a cohort of 199 animals. Statistical tests were carried out by the Department of Medical Statistics, University of Edinburgh.

The large majority of tumours which arose in all genotypes were lymphomas of T-cell origin, and, for simplicity, only this tumour type was used for genetic analysis. Some differences in the tumour spectrum were seen: 12% of *Msh2*<sup>+/+</sup> *p53*<sup>-/-</sup> mice developed sarcoma rather than lymphoma, whilst 40% of *Msh2*<sup>-/-</sup> *p53*<sup>+/+</sup> mice developed intestinal lesions in addition to lymphoma. The distribution of lymphoma origin also varied: *Msh2*<sup>-/-</sup> *p53*<sup>+/+</sup> mice developed thymic and extra-thymic lymphomas in approximately equal proportions, as did *Msh2*<sup>-/-</sup> *p53*<sup>+/-</sup> animals, whereas *Msh2*<sup>+/+</sup> *p53*<sup>-/-</sup> mice succumbed predominantly to thymic lymphomas. Mice homozygously lacking both genes (*Msh2*<sup>-/-</sup> *p53*<sup>-/-</sup>) developed lymphomas which were exclusively thymic, and tumours from *Msh2*<sup>+/-</sup> *p53*<sup>-/-</sup> mice were exclusively extra-thymic.

#### 6.3.2. CGH analysis of chromosomal changes in murine tumours

Results of CGH analysis of lymphomas are presented in Table 14 and Figures 17, 18 and 19. Ten tumours from this series showed signs of chromosomal change. The number of chromosomal locations at which change was scored varied from one to four per tumour. The data (Table 14) indicate that the majority of lesions from *Msh2*<sup>+/+</sup> *p53*<sup>-/-</sup> mice showed at least one chromosomal change (details shown in Figure 18). In contrast, chromosomal anomalies were rarely seen in mice of all other genotypes.

Chromosomes 4 and 15 were over-represented in four tumours, chromosome 11 in three and chromosomes 9 and 14 in two (Figure 18 & 19). Amplifications occurred much more frequently than deletions, with 18 chromosomal amplifications noted (in 9 tumours) and only three deletions. Whole chromosome copy number changes occurred at approximately the same frequency as partial chromosome duplication (Figure 18).

## Figure 17

### Examples of Comparative Genomic Hybridisation analysis of murine lymphomas.

(A) Normal mouse karyotype with DAPI banding, and (B) corresponding control hybridisation of normal female vs normal female mouse DNA, visualised using rhodamine and fluorescein.

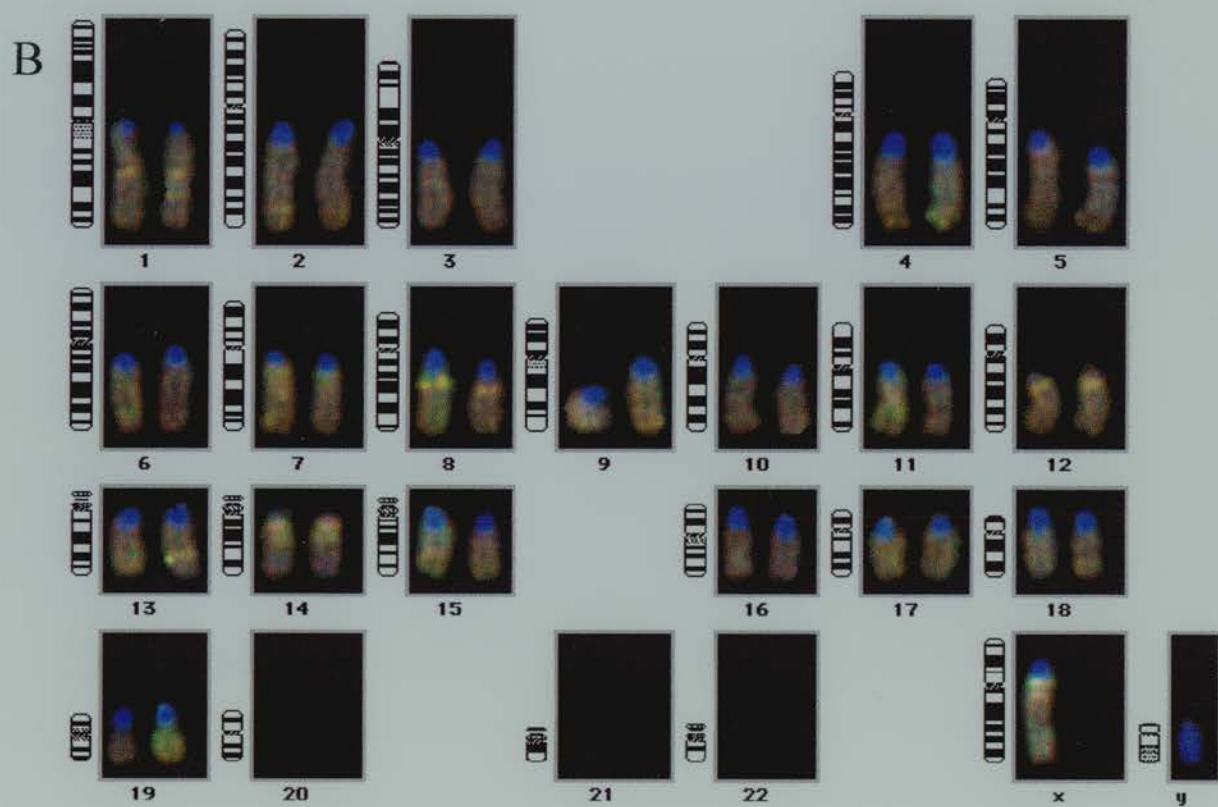
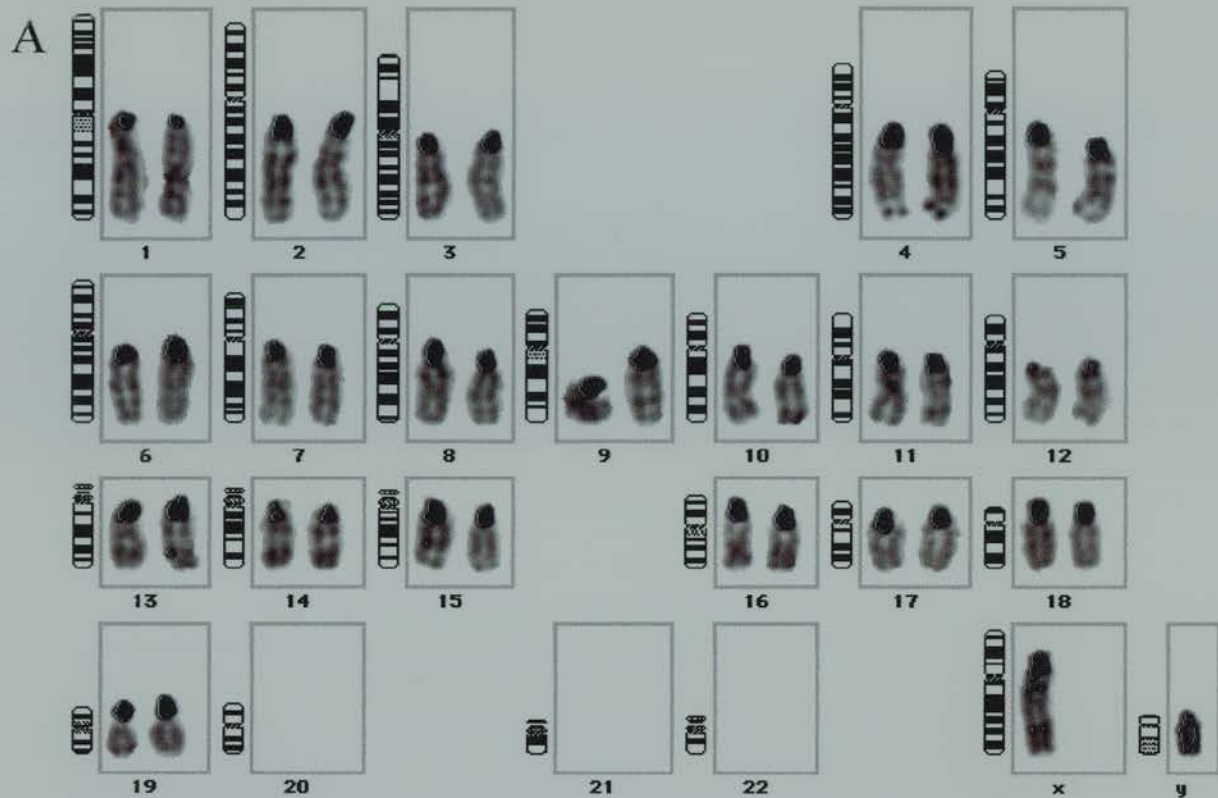
(C) Average ratio profile for 5 metaphases from the above control hybridisation experiment.

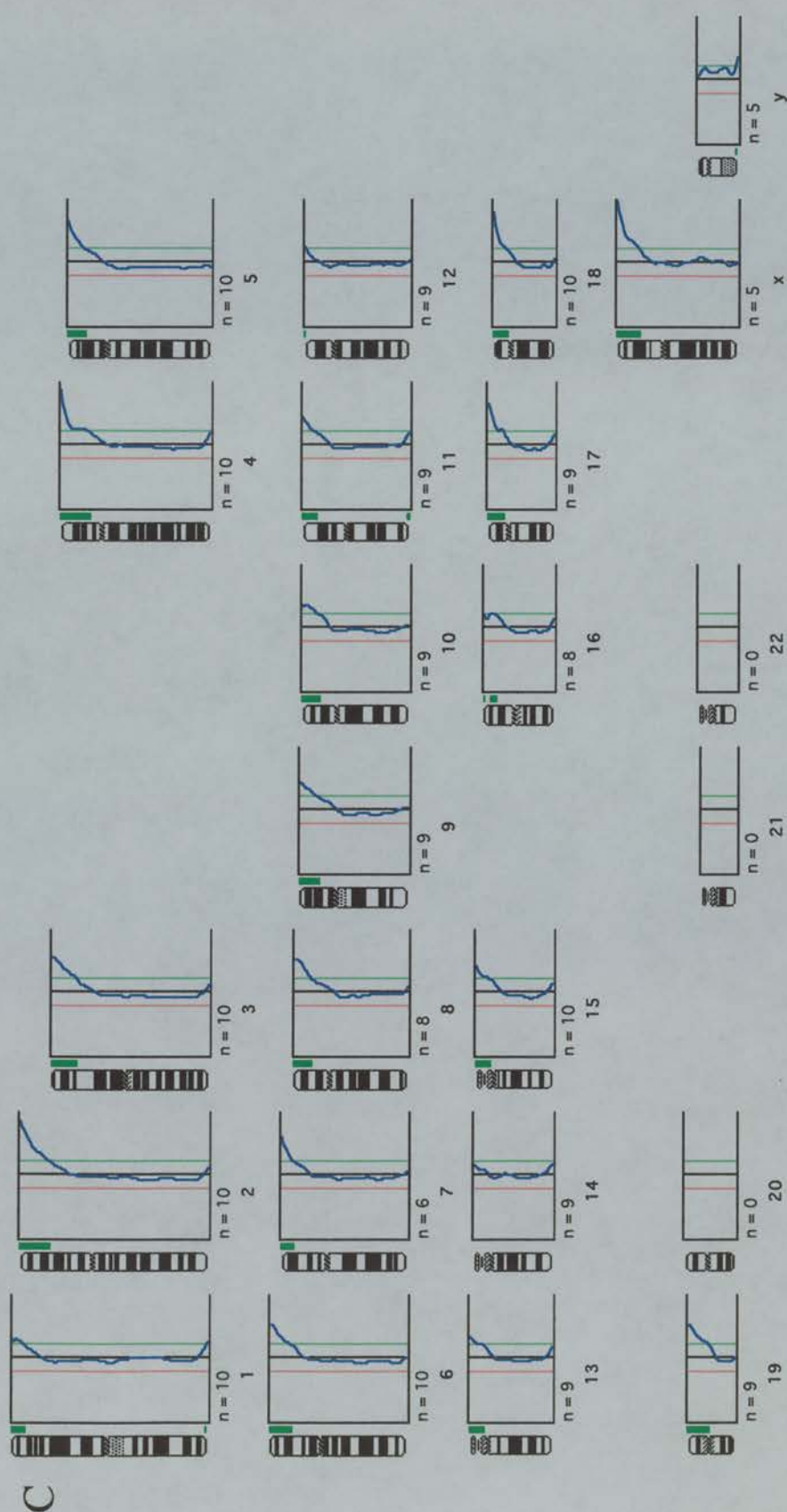
(D) Normal mouse karyotype with DAPI banding, and (E) corresponding hybridisation of normal female mouse DNA (red) vs murine lymphoma DNA (green) from a male *Msh2*<sup>-/-</sup>*p53*<sup>-/-</sup> mouse.

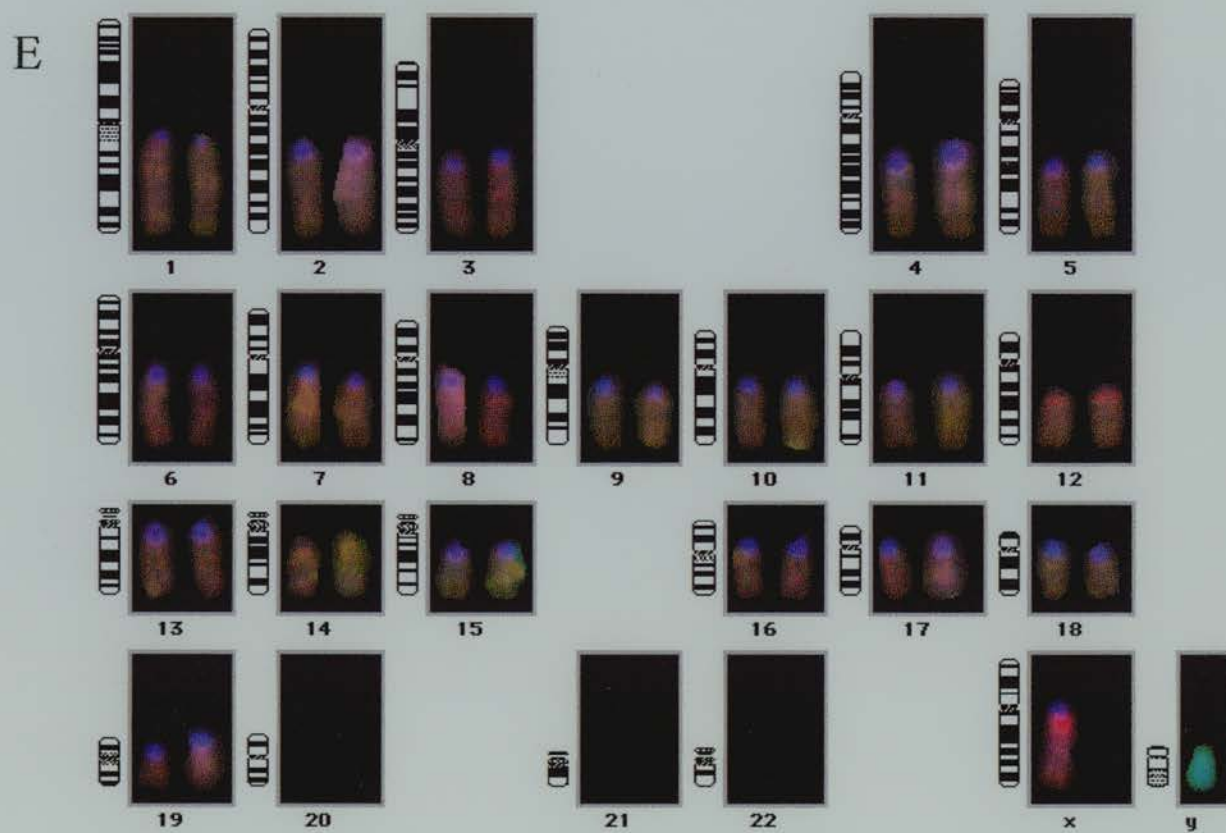
(F) Average ratio profile for 5 metaphases from the hybridisation shown in (D) and (E), showing aneuploid DNA content.

All metaphases were karyotyped manually and imposed upon a human karyotype template for CGH analysis.









F





**Table 14**  
**Diploid and aneuploid DNA content in murine tumours by CGH**

Genotype	No. diploid tumours	No. aneuploid tumours
<i>Msh2</i> +/+ <i>p53</i> -/-	4	6
<i>Msh2</i> -/- <i>p53</i> +/+	5	1
<i>Msh2</i> -/- <i>p53</i> -/-	6	1
<i>Msh2</i> -/- <i>p53</i> +/-	12	1
<i>Msh2</i> +/- <i>p53</i> -/-	5	1

**6.3.3. Analysis of DNA content of murine tumours by flow cytometry**

Overall DNA content was measured by flow cytometry in a subset of tumours different from that used for CGH (Table 15). Results were largely consistent with those obtained by CGH; in this case, all tumours were demonstrated to show near-diploid DNA content except two in which *p53* alone was deficient.

**Table 15**  
**DNA content of tumours from *Msh2*- and *p53*-deficient mice measured by flow cytometry.**

Genotype	No. near-diploid tumours	No. aneuploid tumours
<i>Msh2</i> +/+ <i>p53</i> -/-	5	2
<i>Msh2</i> -/- <i>p53</i> +/+	7	0
<i>Msh2</i> -/- <i>p53</i> -/-	7	0
<i>Msh2</i> -/- <i>p53</i> +/-	7	0

**6.3.4. Microsatellite instability in murine tumours**

Analysis of four microsatellite loci in 20 tumours revealed instability in 11 tumours. Results are presented in Table 16. Microsatellite instability occurred in 9/9 lymphomas with both *Msh2* and *p53* deficiency, even when the *p53* deficiency was heterozygous, compared with only 1/4 tumours deficient in *Msh2* alone. Interestingly, 1/5 tumours with heterozygous loss of *Msh2* and homozygous loss of *p53* showed microsatellite instability at one locus. This tumour was found to have undergone deletion of the wild-type allele of *Msh2*.

**Table 16**  
**Microsatellite instability in tumours from mice deficient in p53, Msh2 or both.**

Genotype	
<i>Msh2</i> <i>p53</i>	No. with MSI at 1 locus or more
+/+   -/-	0/2
-/-   +/+	1/4
-/-   -/-	3/3
-/-   +/-	6/6
+/-   -/-	1/5

**6.3.5.   Analysis of the second allele of p53 in *Msh2*<sup>-/-</sup> *p53*<sup>+/-</sup> mice.**

Animals with the genotype *Msh2*<sup>-/-</sup> *p53*<sup>+/-</sup> develop tumours earlier than those homozygous for *Msh2*-deficiency alone, implying that heterozygous deletion of p53 accelerates tumorigenesis. In order to investigate whether this occurred because of loss of wild-type p53 function, the remaining p53 allele was investigated. Loss of heterozygosity (LOH) was searched for in 7 tumours from animals homozygous for mutation of *Msh2* and heterozygous for mutation of *p53* but was not detected in any tumour. Thus, loss of the second allele of p53 by large deletion appears to be a rare event. Three thymic lymphomas without detectable LOH at the p53 locus were chosen for detailed sequence analysis of the second allele of *p53*. In these tumours, exons 5-9 of *p53*, the region of the gene where mutations are most frequently found (Levine *et al.*, 1991), were sequenced. One tumour carried a G-A (methionine to isoleucine) transition at codon 234, whilst the other two had no detectable mutation.



**Figure 18**

**Chromosomal abnormalities in murine tumours detected by Comparative Genomic Hybridisation**

All duplications and deletions of chromosomal material detected by CGH in murine tumours are depicted in this diagram. Deletions are shown to the left of each chromosome with a thin line, duplications to the right with a thick line, with superimposed thicker lines indicating regions of discrete amplification.



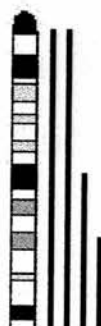
1



2



3



4



5



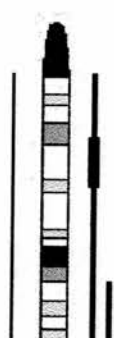
6



7



8



9



10



11



12



13



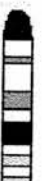
14



15



16



17



18

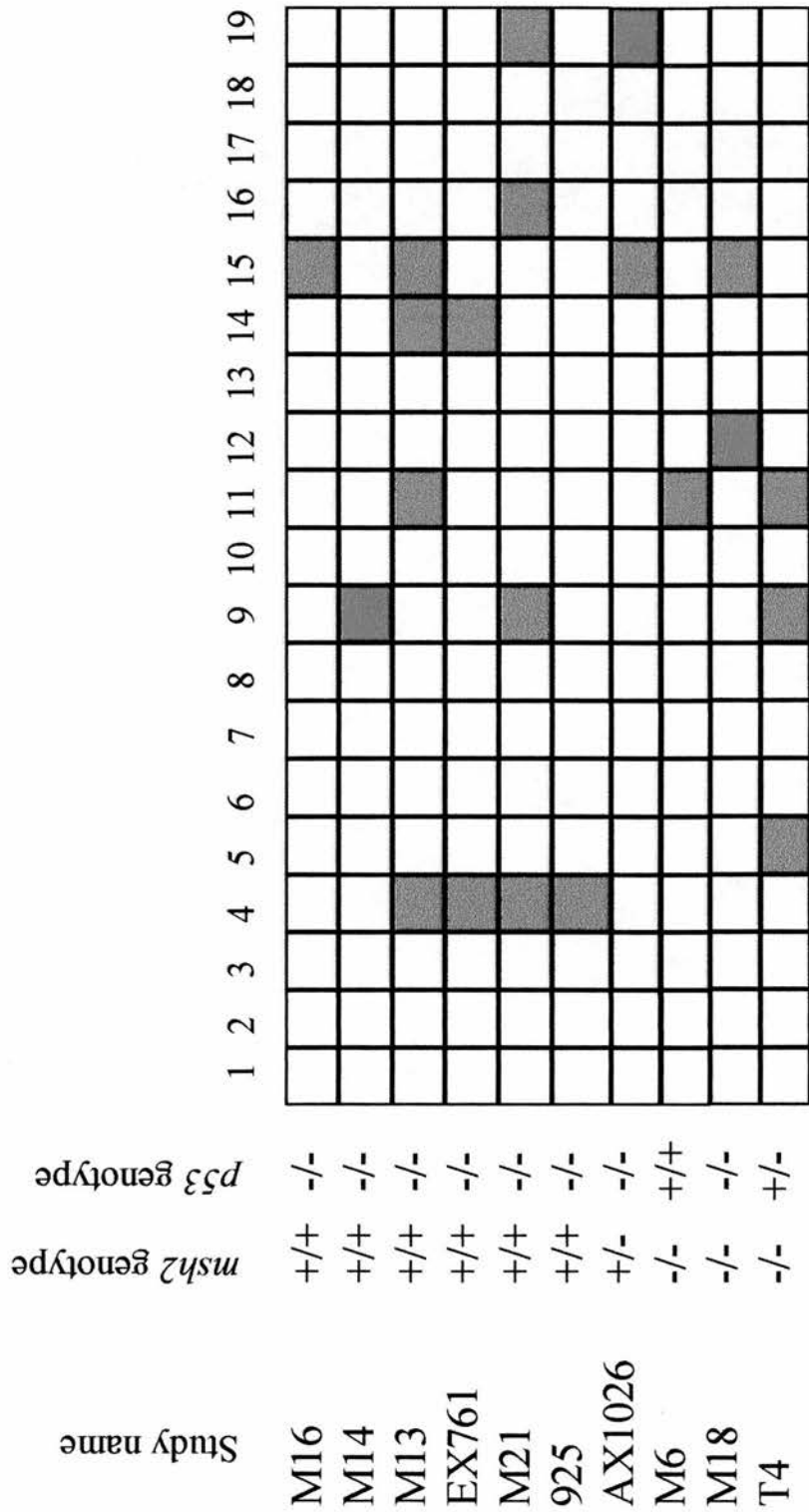


19

**Figure 19**

**Chromosomal abnormalities in aneuploid murine tumours**

This figure indicates the chromosomes affected by the duplications (green) or deletions (red) shown in Figure 18 in each aneuploid tumour. Genotype of *Msh2* and *p53* is indicated.



#### 6.4. Discussion

The phenotypic effects of defective *p53* and *Msh2* in mice have been reported previously, but only recently have mice with combined defects been bred. The generation of mice lacking both *p53* and *Msh2* in this laboratory and elsewhere (Cranston *et al.*, 1997) indicates that the combination of these genetic deletions accelerates tumorigenesis, demonstrating a co-operative effect of the two genes on tumour development. The nature of this synergistic effect has been addressed using analysis of the genetic events known to be associated with each independent defect, microsatellite instability and chromosomal abnormalities, with a view to determining the dominant pathway of genomic instability in tumour development.

The data presented here clearly demonstrate the dominance of the *Msh2*<sup>-/-</sup> phenotype in driving genomic instability in tumours null for both *Msh2* and *p53*. In this study, MSI occurred in all lymphomas in which homozygous loss of *Msh2* was present in combination with any defect of *p53*, whilst the chromosomal anomalies common in *p53*<sup>-/-</sup> tumours were rare in all *Msh2*<sup>-/-</sup> tumours, even those completely lacking *p53*. Furthermore, deficiency of *p53* in *Msh2*<sup>-/-</sup> mice increased to 100% the proportion of tumours in which MSI was detectable compared with *Msh2*<sup>-/-</sup> mice. Thus, whilst the acceleration of tumorigenesis in animals lacking both *Msh2* and *p53* implicates both gene defects in tumour development, the mechanism through which *p53* contributes to tumorigenesis in these mice is distinct from its role in *p53*<sup>-/-</sup> animals, which involves the generation of chromosomal instability.

Why do tumours from animals lacking both *Msh2* and *p53* acquire mutations in microsatellites at a higher rate to that in *Msh2*<sup>-/-</sup> mice but fail to show chromosomal instability? The most plausible explanation is that the microsatellite instability phenotype arises earlier in these mice and thus precedes and preempts chromosomal lesions, resulting, instead, in the development of a tumour in which microsatellite instability is widespread. Chromosomal instability in tumours from mice deficient in *p53* alone may be due to a number of essential functions of *p53*: inability to produce growth arrest at G1 in response to DNA damage, incorrect centrosome formation, lack of a G2 spindle checkpoint or inability to trigger apoptosis in response to DNA damage (discussed in detail in Chapter 1). Mice deficient in *p53* alone display frequent abnormal centrosome formation in a variety of cell types (Fukasawa *et al.*, 1997) suggesting that some chromosomal lesions are a spontaneous and inevitable consequence of *p53*-deficiency, although others may be incurred as a result of exogenous DNA damage. A potent *Msh2*-driven mutagenic pathway which causes multiple mutations in oncogenes and tumour suppressor genes could precede such chromosomal instability if a cell lacks one or more of the functions of *p53* which prevent the clonal expansion of cells harbouring multiple frameshift and point mutations.

The mechanism through which rapid expansion of clones with the microsatellite instability phenotype occurs is likely to be the loss of p53-dependent apoptosis, an increase in the mutation rate at microsatellite sequences, or both. In the first case, *p53*-deficiency could result in failure to respond to apoptotic signals such as *Msh2*-induced mutations which lead to inappropriate expression of oncogenes (Hermeking & Eick, 1994; Lowe *et al.*, 1994), inappropriate expression of factors involved in G1 arrest (Gottlieb & Oren, 1996), or possibly the presence of mismatches themselves (Lee *et al.*, 1995). Widespread *Msh2*-induced mutation may facilitate the disruption of other genes involved in apoptotic pathways, thus speeding the tumorigenic process further. If reduced apoptosis occurs in these tumours, then *Msh2*-deficient tumours in which a high mutation rate is accompanied by p53 inactivity would be expected to contain lower numbers of apoptotic cells than *Msh2*<sup>-/-</sup> tumours. Examination of normal cells from the small intestines of these mice demonstrates that the basal level of apoptosis is low but does not differ from that of other genotypes (Dr N.Toft, personal communication). Levels of apoptosis in lymphoma tissue have not been measured, though have been observed to be generally high in all genotypes (Dr N.Toft, personal communication).

Alternatively, lack of p53 in *Msh2*-deficient tumours may contribute to an increase in the rate of frameshift mutation through direct involvement in repair of mismatches, and the apparent exacerbation of the defective mismatch repair phenotype by lack of p53 could be due to lack of repair of some insertion/deletion mutations. There is evidence that p53 plays a direct role in recognition of insertion/deletion mismatches (Lee *et al.*, 1995), and p53 has been shown to be capable of binding the promoter region of *Msh2*. It is conceivable, then, that p53 is involved in recognition of insertion/deletion mutations and mismatch repair either through its interaction with *Msh2* or independently. The fact that loss of p53 appears to exacerbate the MSI phenotype in *Msh2*<sup>-/-</sup> tumours suggests that its role, if any, in mismatch repair is not exclusively due to interaction with *Msh2*, but it is possible that p53 could partially substitute for *Msh2* where *Msh2* is completely lacking. If this were true, then it is likely to occur only in the absence of *Msh2*, since the rate of point mutations, including insertion/deletion mutations, is not increased in *p53*<sup>-/-</sup> mice (Nishino *et al.*, 1995; Sands *et al.*, 1995).

Chromosomal instability occurs through routes other than the p53-mediated pathway; a recent study suggests that chromosomal instability can arise as a dominant trait independently of p53, at least in human colorectal cancer cells (Lengauer *et al.*, 1997b). It is possible that a further explanation for the lack of chromosomal instability in *Msh2*<sup>-/-</sup>*p53*<sup>-/-</sup> mice is that activation of such a process is favoured in the *p53* null environment, whereas the high rate of frameshift and point mutations within the *Msh2*<sup>-/-</sup> background favour its inactivation. However,



unless evidence is produced to the contrary, the simpler hypotheses based on rates of apoptosis and mutation within such tumours seem more probable. In summary, it appears that the role of p53-deficiency in *Msh2*<sup>-/-</sup> *p53*<sup>-/-</sup> mice is primarily to facilitate the growth cells with *Msh2*-deficiency-type genomic instability, though without further experiment it is not possible to conclude by what mechanism this occurs. Determination of both levels of apoptosis and the rate of insertion/deletion mutation within *Msh2*<sup>-/-</sup> *p53*<sup>-/-</sup> tissue provides the basis for future investigation.

An additional point of interest in these data is the accelerated tumorigenesis in mice heterozygous for *p53* but null for *Msh2*. These lesions develop approximately 20 days later than those in the double nulls, but significantly earlier than in the p53-deficient and *Msh2*-deficient animals, again implying a co-operative interaction between the two pathways in the same manner as the double homozygotes. p53 is conventionally thought to require functional loss of both alleles in order lose its tumour suppressor activity, either through mutation/deletion or through a dominant negative effect, and the majority of tumours developing in *p53*<sup>+/-</sup> mice show loss of the remaining p53 allele (Purdie *et al.*, 1994). However, a dosage effect of p53 has been demonstrated in the intermediate *in vitro* growth rates and radiation- and UV-induced apoptotic responses of cells derived from *p53*<sup>+/-</sup> mice (Clarke *et al.*, 1993; Ms S. Corbet, personal communication), suggesting that loss of just one gene copy of *p53* could permit expression of a rapid growth phenotype in the lymphoid tumours. Loss of heterozygosity of *p53* did not occur in any of the *Msh2*<sup>-/-</sup> *p53*<sup>+/-</sup> tumours examined here, though point mutation of the wild-type allele would be a more likely on an *Msh2*-deficient background. Point mutation of the second allele was only detected in one of three such tumours, which might indicate that heterozygosity of *p53* is sufficient to cause accelerated tumour onset. However, sequencing analysis did not include the whole gene, so mutation cannot be excluded. A functional assay for p53 activity is currently underway in these tumours and should provide a definitive answer.

The appearance of recurrent duplications within chromosomes 4, 11 and 15 in aneuploid tumours suggest the presence in these regions of factors conferring growth advantage. Trisomy 15 has previously been shown to be a frequent event in murine lymphomas (Spira *et al.*, 1979). The regions of amplification are mostly large, and each contain regions of homology with several human chromosomes, making it impossible to delineate any common regions of loss and gain in mouse and man. Analysis of larger numbers of tumours is necessary in order to confirm this finding and determine any other regions of non-random change.

Finally, the aim of studying animal models is to aid our understanding of the tumorigenic process in man. This data presented here clarify the role of *Msh2* and *p53* on lymphoma

development in mice, and clearly demonstrate the dominance of the *Msh2*-associated phenotype as a mechanism of genomic instability in tumorigenesis. Data from human cancers (Chapter 5) was less easy to interpret, probably for a number of reasons: difficulties of interpretation of patterns of p53 expression, the undoubted contribution of other genetic events to the tumour phenotype, and the fact that the major cause of microsatellite instability in sporadic colorectal cancer has yet to be identified and may constitute more than one gene defect, resulting in a range of phenotypes associated with microsatellite instability. Nevertheless, reassessment of the human tumours in the light of data from *Msh2*- and *p53*-deficient mice does indicate a possible similarity between the phenotype of human and murine tumours according to genotype. Combined data from flow cytometry and CGH demonstrated that human cancers with defective p53 (defined as all tumours in which abnormalities of p53 were detected by mutation analysis, loss of 17p or strongly positive immunocytochemistry) and functional mismatch repair were entirely aneuploid, whilst genotypes in which mismatch repair was defective were predominantly diploid regardless of p53 status. Clearly, numbers of human tumours are very small when divided into four genotypes and analysis of a larger series would be essential to confirm this observation. However, data from this study suggest that the observation that p53 abnormalities precede, and probably facilitate, the formation of aneuploid clones in human colorectal tumours (Carder *et al.*, 1993 & 1995) may be dependent upon the presence of functional mismatch repair.

## 6.5. Conclusions

This study has analysed a unique set of tumours arising in mice deficient in the tumour suppressor genes *p53*, *Msh2*, or both. Tumours from mice deficient in p53 alone exhibit frequent chromosomal anomalies, and common regions of amplification on chromosomes 4, 11 and 15 have been identified. A proportion of tumours in mice lacking *Msh2* but with normal *p53* exhibit microsatellite instability, though this is lower than in tumours from *Msh2*<sup>-/-</sup> *p53*<sup>-/-</sup> mice where microsatellite instability occurs ubiquitously. Furthermore, *Msh2*<sup>-/-</sup> *p53*<sup>-/-</sup> tumours rarely display chromosomal lesions. The data suggest that the role of p53 is different in *Msh2*<sup>-/-</sup> *p53*<sup>-/-</sup> and *p53*<sup>-/-</sup> tumours. In the latter, p53 deficiency results in chromosomal instability. In the former, p53 accelerates the development of tumours which evolve primarily through a pathway of *Msh2*-driven multiple point mutations, either through the abolition of p53-dependent apoptosis or through an increase in the rate of point mutation. Comparison with the data from human colorectal tumours obtained in Chapter 5 suggests that a similar relationship may exist between abnormalities of p53 and mismatch repair and chromosomal and microsatellite instability in human cancer, though this remains to be confirmed.

## CHAPTER 7

### SUMMARY

#### 7.1 Summary

This thesis has examined aspects of microsatellite instability, chromosomal instability and defects of p53 from two sources: primary and xenografted human sporadic colorectal cancers, and lymphomas from mice deficient in *p53* and the mismatch repair gene *Msh2*. A number of conclusions can be drawn.

Firstly, the phenotype associated with defects of mismatch repair, microsatellite instability, is present in around one sixth of the Scottish population of sporadic colorectal cancers and is associated with a series of characteristic features: proximal tumour location, lack of abnormality of p53, and, most strikingly, improved patient prognosis. These features resemble those shown by tumours in the familial condition HNPCC, in which a mutated version of hMSH2 or hMLH1 is usually inherited in the germ-line, prompting a search for mutations in hMSH2 in these sporadic tumours. The number of exonic mutations within hMSH2 in sporadic colorectal cancers with microsatellite instability is very small, indicating that hMSH2 is rarely important in the generation of this phenotype in sporadic colorectal cancer. Mutations within repetitive intronic sequences of hMSH2 are frequent but are probably secondary to the mutator phenotype, though they might affect transcriptional efficiency, possibly exacerbating the mutator phenotype. The major cause of this phenotype in sporadic cancer remains to be determined.

Secondly, study of colorectal cancers xenografted into SCID mice demonstrates the microsatellite instability phenotype to be retained through serial passage. Conversely, the phenotype is never acquired by previously stable tumours during passage. This, as well as conservation in the xenograft of a number of other primary tumour characteristics such as ploidy and p53 protein stability provide further support that xenografts are good study models for colorectal cancer.

Thirdly, a striking pattern of chromosomal change is associated with the RER-phenotype in sporadic colorectal tumours, involving deletion of chromosome 8p and 18q and gain of 8q, 13q and 20q. In contrast, cancers in which microsatellite instability occurs do not display this pattern of chromosomal anomaly, although some degree of chromosomal abnormality does occur. This probably reflects the different mechanisms of mutagenesis occurring in each tumour type, though further study may determine whether target genes in these regions are affected by a different spectrum of mutation in each tumour type.

Finally, deficiency of the mismatch repair gene *Msh2* in mice predisposes to lymphoma in which microsatellite sequences are often unstable but chromosomal content is

usually normal, whilst deficiency of *p53* gives rise to lymphoma containing stable microsatellites but abnormal chromosomal content. The combination of both defects accelerates development of chromosomally normal lymphomas in which microsatellite instability is very frequent, indicating the dominant pathway of genomic instability in these tumours to be driven by *Msh2* and suggesting the role of *p53* in tumorigenesis to be dependent upon mismatch repair function. Further study will be required to confirm the observation that human colorectal tumours with equivalent *p53* and mismatch repair status conform to a similar pattern of chromosomal and microsatellite instability.

## 7.2 Future prospects

A series of colorectal cancer xenografts have been characterised for many genetic features including defects of *p53* and of the mismatch repair pathway. These tumours, as well as cancer-prone mice deficient in genes involved in these pathways, should provide a valuable means of determining the effect of new and existing chemotherapeutic drugs in relation to the genetic constitution of the tumour.

At present, little is known about factors determining response to chemotherapy. Chemotherapeutic treatment of colorectal cancer affords an increase in survival of only about 15%, and administration of such treatment largely disregards the biology of the tumour. However, it is becoming clear that many biological factors may influence response to chemotherapy. These include the ability to regulate intracellular drug concentration, achieved in part by expression of the MDR gene product, the nature of the interaction between drug and cellular target and the presence of factors involved in response to damage caused by chemotherapeutic agents. Mismatch repair deficiency has also been shown to confer sensitivity to chloroethylating agents (Liu *et al.*, 1996b) but resistance to temozolomide, alkylating agents and cisplatin (Branch *et al.*, 1993 & 1995; Kat *et al.*, 1993; de Wind *et al.*, 1995; Aebi *et al.*, 1996; Drummond *et al.*, 1996; Fink *et al.*, 1996; Liu *et al.*, 1996b; Mello *et al.*, 1996), at least in some cases through failure to initiate apoptosis (Dr N. Toft, personal communication). Similarly, deficiency of *p53* can result in resistance to certain drugs including the topoisomerase II-inhibitor etoposide (Clarke *et al.*, 1993; Lowe *et al.*, 1993) and doxorubicin (Aas *et al.*, 1996), probably because damage fails to initiate apoptosis in the absence of DNA repair. Moreover, at least some cells survive with incorrectly repaired damage, to generate mutated subclones (Griffiths *et al.*, 1996).

Thus, the combination of genetic defects present within a tumour can be critical in determining response to drug treatment, and characterising the genotype-dependent response of colorectal tumours to current chemotherapy drugs might facilitate the design of treatment appropriate to individual needs. Furthermore, the use of both genetically characterised

xenografted human tumours and murine models in which genetic defects are known precisely may aid our understanding of the pathways by which chemotherapeutic drugs kill tumour cells, thus helping in the design of novel compounds affecting these pathways. Defective gene products could themselves provide targets for new drugs, such as compounds capable of converting mutant p53 to wild-type (Kinzler & Vogelstein, 1993). Use of the resources characterised in this study could provide an invaluable vehicle for such experiments.

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## APPENDIX 1

### REAGENTS AND SUPPLIERS

Reagents are listed in alphabetical order against the name of their supplier(s).

96-well PCR plates; Hybaid Ltd  
 $\alpha^{35}\text{S}$ dATP; Amersham International plc  
 $\alpha^{32}\text{P}$ dCTP; Amersham International plc  
ABC kit; Dako Ltd  
Agarose; Sigma Aldrich Chemical Company  
Alkaline phosphatase substrate kit BCIP/NBT; Vector Laboratories  
Ammonium acetate; Fisher Scientific  
Ammonium persulphate; Severn Biotech Ltd  
Ampicillin; Sigma Aldrich Chemical Company  
Anti-digoxigenin rhodamine; Sigma Aldrich Chemical Company  
 $\beta$ -mercaptoethanol; Sigma Aldrich Chemical Company  
Bacto-agar; Difco Laboratories Ltd  
Bacto-tryptone; Difco Ltd  
BCIP/NBT alkaline phosphatase substrate kit; Dako Ltd  
Bench alcohol; Genta Medical  
Biotin-16 dUTP; Boehringer-Mannheim Ltd  
Biotinylated anti-avidin; Vector Laboratories  
Biotinylated pBR322 standard; Life Technologies Ltd  
Biotinylated rabbit anti-mouse immunoglobulins; Dako Ltd  
Blocking agent; Amersham International plc  
Boric acid; Sigma Aldrich Chemical Company  
Bromophenol blue; Fisher Scientific  
BSA fraction V; Fisher Scientific  
Calcium chloride; Fisher Scientific  
Chick serum; Life Technologies Ltd  
Chloroform; Fisher Scientific  
Cot1 DNA, human and mouse; Life Technologies Ltd  
Cover slips (No.0 & No.1, Chance Propper); Fisher Scientific  
'Decon' detergent; Fisher Scientific  
Deionised distilled water (DDW); Elga Ltd  
DEPC; Sigma Aldrich Chemical Company  
Diaminobenzidine (DAB); Sigma Aldrich Chemical Company  
Diaminoethanetetra-acetic acid (EDTA); Sigma Aldrich Chemical Company  
Diaminophenolindole (DAPI); Boehringer-Mannheim Ltd  
Digoxigenin-11-dUTP; Boehringer-Mannheim Ltd  
Digoxigenin-labelled lambda DNA standard; Sigma Aldrich Chemical Company  
Dimethyl sulfoxide (DMSO); Sigma Aldrich Chemical Company  
Dithiothreitol (DTT); Sigma Aldrich Chemical Company  
DNA mass ladder; Life Technologies Ltd  
DNA molecular weight marker V; Boehringer-Mannheim Ltd  
DNA molecular weight marker 1 kilobase DNA ladder; Life Technologies Ltd  
DNA molecular weight marker  $\phi$ X174 RF DNA/HaeIII; Life Technologies Ltd

DNA polymerase I; Life Technologies Ltd  
 DNase; Boehringer-Mannheim Ltd  
 dNTPs; Pharmacia Biotechnologies Ltd  
 Do7 antibody; Dako Ltd  
 Dynabeads M280 streptavidin; Dynal Ltd  
*EcoRI* + buffer; NBL Gene Science Ltd  
 Ethanol; Hayman Ltd  
 Ethidium bromide; Sigma Aldrich Chemical Company  
 Fluorescein-avidin DCS; Vector Laboratories  
 Foetal bovine serum; Sigma Aldrich Chemical Company  
 Formaldehyde solution; Fisher Scientific  
 Formamide; Fisher Scientific  
 $\gamma$ <sup>33</sup>PdATP; Amersham International plc  
 $\gamma$ -methacryloxypropyltrimethoxysilane; Sigma Aldrich Chemical Company  
 'Gel Slick'; AT Biochem  
 Giemsa; Sigma Aldrich Chemical Company  
 Glacial acetic acid; Fisher Scientific  
 Glycerol; Sigma Aldrich Chemical Company  
 Glucose; Fisher Scientific  
 Hybond N+; Amersham International plc  
 Hydrochloric acid; Fisher Scientific  
 Hydrogen peroxide; Sigma Aldrich Chemical Company  
 Ion-exchange resin beads; Bio-Rad Laboratories Ltd  
 Imidazole; Sigma Aldrich Chemical Company  
 Immuno-Check alignment fluorospheres; Coulter Electronics Ltd  
 'Instagel' 40% 19:1 acrylamide:bis acrylamide solution; Severn Biotech Ltd  
 Iso-amyl alcohol; Fisher Scientific  
 Isopropanol; Fisher Scientific  
 Isopropyl  $\beta$ -D-thiogalactopyranoside (IPTG); Sigma Aldrich Chemical Company  
 Klenow fragment; Amersham International plc  
 Kodak X-OMAT autoradiography film; Amersham International plc  
 $\lambda$  HindIII marker; Life Technologies Ltd  
 L-glutamine; Life Technologies Ltd  
 Library efficiency DH5 $\alpha$  competent cells; Life Technologies Ltd  
 Low melting point agarose; Life Technologies Ltd  
 Magnesium chloride; Sigma Aldrich Chemical Company  
 Magnesium Sulphate; Sigma Aldrich Chemical Company  
 MDE gel; AT Biochem  
 Methanol; Fisher Scientific  
 Microscope slides 'Select' (Chance Proper); Fisher Scientific  
 N,N,N',N'-tetramethylethylenediamine (TEMED); Severn Biotech Ltd  
 Nonidet P40; Fisher Scientific  
 Normal rabbit serum; Life Technologies Ltd  
 Oligonucleotides; Oswel DNA, Cruachem and VHBio  
 pAB1801; Dako Ltd  
 Phosphate buffered saline (PBS); Life Technologies Ltd  
 Phytohaemmagglutinin (M-form); Murex Diagnostics Ltd and Sigma Aldrich Chemical Company  
 Potassium chloride; Fisher Scientific  
 Potassium dihydrogen orthophosphate; Fisher Scientific

'Prime-It' RmT random primer labelling kit; Stratagene  
 Propan-2-ol; Fisher Scientific  
 Propidium iodide; Sigma Aldrich Chemical Company  
 Proteinase K; ICN Biomedicals Ltd  
*Pst*I + buffer; NBL Gene Science Ltd  
*Pvu*II + buffer; Life Technologies Ltd  
 QuickPrep Total RNA Extraction Kit; Pharmacia Biotechnologies Ltd  
 Quick Spin columns G50 (fine); Boehringer-Mannheim Ltd  
 RNase A; Sigma Aldrich Chemical Company  
 RPMI medium (Dutch modification); Life Technologies Ltd  
*rTth* DNA polymerase, XL, 3.3x XL buffer and Mg(OAc)<sub>2</sub> from XL PCR kit; Perkin Elmer Ltd  
 Sequenase version 2.0 DNA polymerase and all other sequencing reagents; Amersham International plc  
 Sequencing apparatus; Bio-Rad Ltd  
*Sfe*I + buffer; Life Technologies Ltd  
 Silver nitrate; Fisher Scientific  
 Silver stain kit; Bio-Rad Ltd  
*Sma*I + buffer; Boehringer-Mannheim Ltd  
 Sodium carbonate (decahydrate); Fisher Scientific  
 Sodium chloride; Fisher Scientific  
 Sodium citrate; Fisher Scientific  
 Sodium dodecyl sulphate (SDS); ICN Biomedicals Ltd  
 Sodium hydroxide; Fisher Scientific  
 Sodium dihydrogen orthophosphate; Fisher Scientific  
 Sodium thiosulphate; Fisher Scientific  
 Spermidine; Sigma Aldrich Chemical Company  
 Spermine tetrahydrochloride; Sigma Aldrich Chemical Company  
 Spin Columns MicroSpin S-200 HR; Pharmacia Biotechnologies Ltd  
 Streptavidin alkaline phosphatase; Boehringer-Mannheim Ltd  
 Streptavidin alkaline phosphatase anti-digoxigenin; Boehringer-Mannheim Ltd  
 Sucrose; Fisher Scientific  
 Superscript Preamplification System; Life Technologies Ltd  
 T4 DNA ligase; Life Technologies Ltd  
 T4 polynucleotide kinase + buffer; Life Technologies Ltd  
 TA cloning kit; Invitrogen BV  
 ThermoSequenase radio labelled terminator cycle sequencing kit, including all enzymes, termination master mixes and reaction buffer; Amersham International plc  
 Thermostable DNA polymerase + buffer IV; Advanced Biotechnologies Ltd  
 Tissue culture plasticware; Costar Ltd  
 Tris; Sigma Aldrich Chemical Company  
 Trisodium citrate; Sigma Aldrich Chemical Company  
 TRIzol reagent; Life Technologies Ltd  
*Tru*II + buffer; MBI Fermentas  
 Trypsin; Sigma Aldrich Chemical Company  
 Trypsin Inhibitor; Sigma Aldrich Chemical Company  
 Tween 20; Fisher Scientific  
 Quick Spin columns G50 (fine); Boehringer-Mannheim Ltd  
 Urea; Fisher Scientific

'Vectashield' mounting medium; Vector Laboratories  
Water-saturated phenol; Rathburn Chemicals  
Whatman paper; Whatman Ltd  
Wizard miniprep kit; Promega Ltd  
Xylene; Fisher Scientific  
Xylene cyanol FF; Sigma Aldrich Chemical Company  
Yeast extract; Difco Laboratories Ltd

## APPENDIX 2

### SUPPLIERS' ADDRESSES

Advanced Biotechnologies Ltd  
Units B1-B2  
Longmead Business Centre  
Blenheim Road  
Epsom  
Surrey  
KT19 9QQ

Amersham International plc  
Amersham Place  
Little Chalfont  
Buckinghamshire  
HP7 9NA

AT Biochem  
30 Spring Mill Drive  
Malvern  
PA 19355  
USA

Boehringer-Mannheim UK (Diagnostics and Biochemicals) Ltd  
Bell Lane  
Lewes  
E. Sussex  
BN7 1LG

Bio-Rad Laboratories Ltd  
Bio-Rad House  
Maylands Avenue  
Hemel Hempstead  
Hertfordshire  
HP2 7TD

Costar UK Ltd  
10 The Valley Centre  
Garden Road  
High Wycombe  
Buckinghamshire  
HP13 6EQ

Coulter Electronics Ltd  
Luton  
Bedfordshire

Cruachem Ltd  
Todd Campus  
West of Scotland Science Park  
Aire Road  
Glasgow  
G20 OUA

Dako Ltd  
16 Manor Courtyard  
Hughenden Avenue  
High Wycombe  
Buckinghamshire  
HP13 5RE

Difco Laboratories Ltd  
Central Avenue  
East Molesey  
Surrey  
KT805E

Dynal (UK) Ltd  
26 Grove Street  
New Ferry  
Wirral  
L62 5AZ

Elga Ltd  
High Street  
Lane End  
Buckinghamshire  
HP14 3JH

Fisher Scientific UK  
Bishop Meadow Road  
Loughborough  
Leicestershire  
LE11 ORG

Genta Medical  
Marston Business Park  
Rudgate  
Tockwith  
York  
YO5 8QF



Hayman Ltd  
70 Eastways Industrial Park  
Witham  
Essex  
CM8 3YE

Hybaid Ltd  
111-113 Waldegrave Road  
Teddington  
Middlesex  
TW11 8LL

ICN Biomedicals Ltd  
Unit 18  
Thame Park Business Centre  
Wenman Road  
Thame  
Oxfordshire  
OX9 3XA

Invitrogen BV  
De Schelp 12  
9351 NV Leek  
The Netherlands

Life Technologies Ltd  
3 Fountain Drive  
Inchinnan Business Park  
Paisley  
PA4 9RF

MBI Fermentas  
Supplied by Immunogen International Ltd  
Colima Avenue  
Sunderland Enterprise Park  
Sunderland  
Tyne and Wear  
SR5 3XB

Murex Diagnostics Ltd  
Central Road  
Temple Hill  
Dartford  
DA1 5LR

NBL Gene Science Ltd  
South Nelson Industrial Estate  
Cramlington  
Northumberland  
NE23 9HL

Oswel DNA  
Lab 5005  
Medical and Biological Sciences Building  
University of Southampton  
Boldrewood  
Bassett Crescent East  
Southampton  
SO16 7PX

Perceptive Scientific International  
Halladale  
Lakeside  
Chester Business Park  
Wrexham Road  
Chester  
CH4 9QT

Perkin-Elmer Ltd  
Post Office Lane  
Beaconsfield  
Buckinghamshire  
HP9 1QA

Pharmacia Biotechnologies Ltd  
Davy Avenue  
PO Box 100  
Knowlhill  
Milton Keynes  
MK5 8PB

Rathburn Chemicals Ltd  
Walkerburn Scotland  
EH46 6AU

Severn Biotech Ltd  
Unit 2  
Park Lane  
Kidderminster  
Worcestershire  
DY11 6TJ

Sigma-Aldrich Company Ltd  
Fancy Road  
Poole  
Dorset  
BH17 7NH

Stratagene Ltd  
Cambridge Innovation Centre  
140 Cambridge Science Park  
Milton Road  
Cambridge  
CB4 4GF

Vector Laboratories  
16 Wulfric Square  
Bretton  
Peterborough  
PE3 8RF

VHBio Ltd  
PO Box 7  
Gosforth  
Newcastle upon Tyne  
NE3 4DB

Vysis (UK) Ltd  
Rosedale House  
Rosedale Road  
Richmond  
Surrey  
TW9 25Z

Whatman International Ltd  
St Leonard's Road  
20/20 Maidstone  
Kent  
ME16 OLS

## APPENDIX 3

### DNA MOLECULAR WEIGHT MARKER SIZES

Band sizes of markers in base pairs:

1kb ladder	Marker V	DNA mass ladder	φX174 RF DNA/ <i>Hae</i> III	λ DNA/ <i>Hind</i> III
12216	587	2000	1353	23130
11198	540	1200	1078	9416
10180	504	800	872	6557
9162	458	400	603	4361
8144	434	200	310	2322
7126	267	100	281	2027
6108	234		271	564
5090	213		234	125
4072	192		194	
3054	184		118	
2036	124		72	
1636	[123]			
1018	104			
517	89			
506	[80]			
396	[64]			
344	[57]			
298	[51]			
220	[21]			
201	[18]			
154	[11]			
134	[8]			
75				

Numbers in brackets [ ] indicate bands that cannot usually be distinguished.

## APPENDIX 4

### ABBREVIATIONS

ALF	Automated Laser Fluorography
APS	Ammonium persulphate
bp	Base pairs
BSA	Bovine serum albumin
CAD	Trifunctional enzyme carbamoyl-P synthetase, aspartate transcarbamylase, dihydroorotase
CCD	Charged coupled device
cDNA	copy deoxyribonucleic acid
CIN	Chromosomal instability
DAB	3,3'-Diaminobenzidine tetrahydrochloride
DAPI	4,6-diamidino-2-phenylindole
dATP	deoxyadenine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanine triphosphate
dTTP	deoxythymidine triphosphate
dITP	deoxyinosine triphosphate
DEPC	Diethyl pyrocarbonate
DMSO	dimethylsulphoxide
DNA	Deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
ddNTP	dideoxynucleotide triphosphate
DDW	Distilled deionised water
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid (disodium salt)
FAP	Familial Adenomatous Polyposis
GTBP	G-T binding protein
hMLH1	Human MutH homologue
hMSH2	Human MutS Homologue
hPMS1	Human post-meiotic segregation homologue 1
hPMS2	Human post-meiotic segregation homologue 2
HNPCC	Hereditary Non-Polyposis Colorectal Cancer
IPTG	Isopropyl $\beta$ -D-thiogalactopyranoside
MDE	modified acrylamide (trade name)
MNNG	N-methyl-N'-nitro-N-nitrosoguanine
MNU	N-methyl-N-nitrosurea
MSI	Microsatellite instability
mRNA	Messenger ribonucleic acid
NRS	Normal rabbit serum
OD	Optical density
PALA	N-(phosphonacetyl)-L-aspartate
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
Pu	Purine
Py	Pyrimidine
RER+	Replication error positive

RER-	Replication error negative
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
SAP	Shrimp alkaline phosphatase
SCID	Severe combined immuno-deficient
SDS	Sodium dodecyl sulphate
STR	Short tandem repeat
SSC	Salt/sodium citrate buffer
SSCP	Single-stranded conformational polymorphism
TBE	Tris-borate-EDTA
TE	Tris-EDTA
TEMED	N,N,N',N'-tetramethylethylenediamine
TGF $\beta$	Transforming growth factor $\beta$
TGF $\beta$ RII	Transforming growth factor $\beta$ receptor subunit II
UDDW	Ultrapure distilled deionised water
UV	Ultra violet



# Appendix 5b. Details of patients and tumours in this study

Patient	Xeno. name	Pathol- ogy ref.	Age	Sex	Site	Side	Type	Dukes	p53 IHC	RER status	Chap.3 ref.no.	Chap. 4	CGH
1		90 09283	76	F	S	L	AC	A	NA	N			
2		90 14053	61	F	R	L	MUC	C	N	N	38		
3		90 18573	74	F	A	R	AC	B	P	N			
4		90 22158	67	M	R	L	MUC	B	N	N			
5		90 24901	80	F	C	R	AC	C	N	N			
6		90 09966	86	F	A	R	MUC	C	N	N			
7		90 14113	63	F	S	L	AC	C	N	N			
8		90 20472	56	F	S	L	MUC	C	N	S1	55		
9		90 22641	43	F	C	R	AC	C	N	RER+	82		
10		91 16209	37	F	R	L	AC	B	P	S1	56		
11		90 12596	80	F	D	L	AC	C	P	N			
12		90 15056	64	F	S	L	AC	A	P	N			
14		90 22704	77	F	S	L	MUC	C	P	N			
15		90 13702	73	F	R	L	AC	B	P	N			
16		90 13557	47	M	D	L	MUC	C	N	N			
17		90 16283	74	F	R	L	MUC	C	N	N			
18		90 21676	78	F	A	R	MUC	B	P	N			
19		90 26744	85	M	S	L	AC	C	P	N	57		
20		90 14018	69	M	S	L	MUC	B	P	RER+	83		
21		90 17564	73	M	S	L	AC	C	P	N	39		
22		90 21776	77	F	R	L	AC	B	P	N	58		
23		91 13546	74	M	S	L	AC	B	P	N			
24		NA	93	F	A	R	AC	B	N	N			
25		90 24900	47	F	D	L	AC	B	P	N			
26		91 00184	64	F	S	L	AC	B	P	N	59		
27		91 06883	73	M	T	R	MUC	B	P	RER+	30		
28		90 23997	70	F	R	L	AC	C	P	N	60		
29		90 25170	58	M	R	L	AC	B	N	N			
30		91 00385	75	F	A	R	MUC	C	N	RER+	61		
31		90 23997	80	M	R	L	AC	C	P	N			
32		90 25251	64	M	R	L	AC	B	P	N			
33		91 00765	63	M	R	L	MUC	C	N	RER+	84		
34		90 24393	82	F	T	R	AC	B	N	N			
35		90 27079	63	F	T	R	AC	C	N	N			
36		91 01206	69	M	D	L	AC	B	P	N	31		
37		90 24672	68	M	C	R	AC	B	P	N			
38		90 28426	67	M	R	L	AC	A	N	N			
39		91 03489	79	M	S	L	AC	B	P	N			
40		91 07197	73	M	A	R	AC	B	P	N			
41		91 13235	84	F	S	L	AC	C	P	S1	32		
42	JOCR	92 00888	37	M	R	L	MUC	C	P	N	40	yes	yes
43		91 07239	74	M	C	R	MUC	B	N	N	33		
44		91 13273	80	F	R	L	MUC	C	N	N			
45	CORU	91 07283	41	M	S	L	AC	C	N*	N		yes	yes
46		91 16090	76	M	D	L	MUC	B	N	RER+	34		
47	JOLO	91 08679	57	M	S	L	AC	B	P	N		yes	yes
49		91 19323	71	F	R	L	AC	C	P	N			
50		91 21073	42	F	R	L	AC	C	P	N			
51		92 02461	68	M	S	L	AC	B	P	N			
52		92 07513	65	M	R	L	AC	B	P	N			
53		91 21510	67	F	A	R	MUC	C	P	Y	62		
54		91 25643	53	F	S	L	AC	C	N	N	63		
56		92 19204	74	F	R	L	NA	B	P	N			
57	EVMU	92 16518	49	F	C	R	MUC	B	N	Y4	35	yes	yes
58		92 23361	37	F	S	L	AC	C	P	N			
60		92 18759	73	M	C	R	AC	B	P	N			
61		92 21270	81	M	T	R	AC	C	P	N			
62		92 16946	63	F	R	L	AC	B	P	N			
63		92 23420	79	M	C	R	AC	B	P	N			
64		92 17967	71	F	S	L	AC	B	P	N			
65		92 19037	79	F	A	R	AC	B	P	RER+	85		

Appendix 5b. Details of patients and tumours in this study

Patient	Xeno. name	Pathol- ogy ref.	Age	Sex	Site	Side	Type	Dukes	p53 IHC	RER status	Chap.3 ref.no.	Chap. 4	CGH
66		92 21280	79	M	A	R	AC	C	N	N			
67		92 17035	68	M	R	L	AC	A	P	RER+	86		
68		92 23518	74	M	C	R	AC	B	P	N	36		
69		92 18324	63	M	S	L	AC	B	P	N			
70		92 19066	64	M	A	R	AC	C	N	N			
71		92 21973	88	M	D	L	AC	B	P	N			
72		92 17066	66	M	T	R	AC	B	P	N			
73		92 24208	81	M	C	R	AC	A	P	N			
75		92 19066	see#70		C	R	MUC	C?	P	N			
76		92 24208	see#73		C	R	AC	B	P	N	41		
77		92 18557	94	F	A	R	AC	A	N	N			
78		92 18409	83	F	R	L	AC	C	P	N	87		
80		91 23749	65	F	S	R	AC	C	N	N			
81		91 16959	76	F	C	R	AC	C	P	N	42		
82		91 22448	84	M	A	R	AC	A	N	N			
83		92 08987	79	F	R	L	AC	A	N	N			
84		92 10402	82	M	R	L	AC	A	P	N			
85		92 16621	46	F	R	L	AC	B	P	N			
89		92 11405	67	F	T	R	AC	C	P	N	65		
90		92 17710	81	F	C	R	AC	B	P	N	44		
91		91 18129	69	F	S	L	AC	C	N	N			
92		92 07720	62	M	T	R	AC	B	N	N	45		
93		92 09802	70	M	A/T	R	AC	B	N	N			
94		92 11777	68	M	S	L	AC	C	P	N			
95		91 21208	88	M	R	L	MUC	B	N	N	46		
96	ELWI	92 07753	84	M	C	R	AC	C	P	N		yes	yes
97		92 09859	42	M	R	L	AC	C	N	RER+	66		
98	SAGR	92 15601	39	F	C	R	MUC	B	N	N			yes
99		91 21352	71	F	A	R	AC	C	N	RER+	88		
100		92 08746	68	F	R	L	AC	A	P	N			
101		92 10009	79	M	C	R	AC	B	P	N			
102		92 26072	74	F	A	R	AC	B	P	N			
103		92 24371	49	M	R	L	AC	B	P	N	47		
104	ROPE	92 12230	83	M	C	R	AC	C	P	N	48	yes	yes
105		90 27894	63	F	R	L	AC	C	N	N			
106		90 21258	78	F	C	R	AC	C	N	N	49		
107		93 00741	56	F	R	L	AC	B	N	N	67		
108		90 23602	61	M	R	L	AC	C	N	N			
109		90 27183	84	F	D	L	AC	C	P	N			
110		91 03510	73	M	R	L	MUC	C	P	N			
111		90 10048	67	M	R	L	MUC	C	N	N			
112		93 03406	37	F	S	L	AC	C	P	N			
113		93 12255	80	M	C	R	NA	C	P	S1	50		
114		90 19430	56	F	A	R	MUC	B	N	RER+	51		
115		90 10965	65	M	C	R	MUC	B	P	N			
116		93 09806	73	M	S	L	AC	A	N	N	68		
118		91 00982	88	F	A	R	MUC	B	N	RER+	89		
119		90 20135	90	F	R	L	AC	A	P	N	52		
120		90 11629	78	M	T	R	AC	B	P	N			
121		93 09932	63	M	R	L	AC	A	N	N	98		
122		93 12563	71	M	T	R	MUC	B	P	S1	53		
123		93 12923	82	F	NK	L	MUC	B	N	N			
124		91 03489	see#39		R	L	AC	B	P	N			
128		90 18647	61	F	R	L	AC	C	P	N			
130	EDLA	91 08727	49	M	A	R	AC	C	P	N	69	yes	yes
131		90 13666	55	M	R	L	MUC	C	N	N			
cc1		89 19963	63	F	T	R	MUC	B	N	RER+	1		
cc7		901009	63	F	R	L	MUC	C	N	S1	2		
cc11		90 13455	59	M	S	L	AC	B	P	N			
cc13		90 14812	71	F	R	L	AC	B	N	N			
cc17		904626	67	F	R	L	AC	C	N	N			

**Appendix 5b.** Details of patients and tumours in this study

Patient	Xeno. name	Pathol- ogy ref.	Age	Sex	Site	Side	Type	Dukes	p53 IHC	RER status	Chap.3 ref.no.	Chap. 4 CGH
cc19		903349	78	F	A	R	MUC	B	N	N		
cc21		89 29891	40	F	C	R	AC	C	NA	N		
cc25		90 20547	69	M	C	R	MUC	B	N	RER+ 3		
cc27		90 20904	see#120		C	R	AC	A	N	N		
cc29		90 21237	68	M	R	L	MUC	C	N	N		
cc31		90 21524	69	F	A	R	AC	B	P	N		
cc33		90 21677	74	F	S	L	AC	B	N	N		
cc37		90 26513	80	F	A	R	AC	B	P	N	14	
cc39		90 25215	80	F	C	R	AC	B	P	N		
cc41		90 26578	61	M	T	R	AC	B	P	N		
cc43		90 26644	70	F	R	L	AC	A	N	N		
cc49		90 14688	84	M	R	L	AC	B	P	N		
cc51		90 6294	57	M	S	L	AC	C	P	N		
cc53		90 13758	79	F	S	L	AC	CD	N	N		
cc55		9011167	75	F	A	R	MUC	CD	N	N		
cc57		907740	72	F	A	R	AC	C	P	N		
cc59		8818014	87	F	C	R	AC	C?D	NA	N		
cc61		8910961	59	F	R	L	AC	C	P	N		
cc61		893942	55	M	S	L	AC	A	P	N		
cc65		893740	44	F	R	L	AC	B	N	N		
cc67		893719	80	F	R	L	AC	B	P	N		
cc69		8815396	56	M	S	L	AC	C	NA	N		
cc71		893362	77	F	R	L	AC	C	N	N		
cc73		893157	49	M	R	L	AC	B	N	S1 4		
cc75		892292	57	F	C	R	AC	CD	P	N 5		
cc77		891812	57	F	S	L	AC	B	P	N		
cc79		89769	81	F	R	L	AC	A	P	N		
cc81		8829525	75	F	C	R	MUC	C	N	N		
cc83		8829379	49	M	R	L	AC	B	N	N		
cc85		8826932	68	F	S	L	AC	B	P	RER+ 6		
cc87		8826553	70	M	D	L	AC	CD	N	N		
cc89		8826457	68	M	R	L	AC	C	N	N		
cc91		8823757	82	F	S	L	AC	C	P	N		
cc93		8824479	75	F	AA	R	AC	BA	P	N		
cc101		9101896	68	M	R	L	AC	C	P	N		
cc103		9103220	74	M	C	R	MUC	B	P	S1 7		
cc105		9103437	60	M	R	L	AC	B	N	N		
cc107		91+G482	72	M	R	L	AC	C	P	N		
cc109		9103541	90	F	R	L	AC	C	P	S1		
cc111		915034	76	F	S	L	AC	C	P	RER+		
cc113		914328	77	M	A	R	MUC	B	N	N		
cc115		914818	71	F	R	L	AC	B	N	N		
cc117		913751	84	M	R	L	AC	A	P	N		
cc119		9104009	69	M	R	L	AC	C	N	N		
cc123		9105398	75	F	A	R	AC	B	P	S1 9		
cc129		9106001	82	F	S	L	AC	B	P	N		
cc131		9106266	68	M	R	L	MUC	C	N	N		
cc133		9106317	69	M	R	L	MUC	B	P	S1 11		
cc135		9106383	80	M	S	L	AC	CD	P	N		
cc137		9106865	77	M	S	L	AC	B	P	N		
cc139		9107205	70	F	R	L	AC	A	P	N 12		
cc141		9107952	63	M	R	L	AC	C	P	N		
cc143		9107972	68	M	R	L	AC	C	P	N		
cc145		919806	53	F	S	L	AC	C	P	N		
cc147		919761	65	F	C	R	AC	C	P	N		
cc153		9118416	78	M	S	L	MUC	B	P	N 15		
cc155	CADU	9119828	95	F	S	L	AC	B	P	N		yes yes
cc157		9120214	74	M	R	L	AC	B	P	N		
cc161		9109364	72	F	A	R	AC	B	N	N		
cc165		9110345	74	M	S	L	AC	C	P	N		
cc167		9110738	76	F	R	L	AC	B	P	N		

**Appendix 5b.** Details of patients and tumours in this study

Patient	Xeno. name	Pathol- ogy ref.	Age	Sex	Site	Side	Type	Dukes	p53 IHC	RER status	Chap.3 ref.no.	Chap. 4	CGH
cc169		9111087	84	F	R	L	MUC	C	P	N			
cc171		9111612	79	M	C	R	AC	B	P	S1	16		
cc175		9112153	85	F	C	R	MUC	C	N	N			
cc177	GRZBO	9112386	87	F	U	L	AC	A	N	N		yes	yes
cc179	MEMCG	9112695	64	F	A	R	MUC	B	N	RER+	17	yes	yes
cc181		9111273	28	M	R	L	AC	D	N	N			
cc183	THLE	9117951	67	M	R	L	AC	C	P	N		yes	yes
cc185		9118936	76	F	C	R	MUC	B	P	RER+	19		
cc187		9107351	34	F	R	L	AC	C	P	N			
cc189		9120281	79	F	R	L	AC	B	N	N			
cc191	PAMCI	9121142	65	F	R	L	AC	C	P	N		yes	yes
cc193	WITO	9119029	76	M	R	L	AC	A	N*	S1	20	yes	yes
cc195		9121014	72	F	T	R	AC	C	N	N	21		
cc197		9120182	73	M	C	R	AC	C	N	N			
cc199		9116028	83	F	R	L	AC	B	P	N			
cc201		923180	81	M	A	R	AC	B	P	RER+	22		
cc203		922676	72	M	R	L	AC	C	N	N	23		
cc205	JEGA	923835	69	F	A	R	AC	B	P	N			yes
cc207		9121842	60	M	T	R	AC	C	N	S1	24		
cc209		9122887	70	M	R	L	AC	B	P	N			
cc213		9124370	62	M	S	L	AC	B	P	N			
cc215		9126854	62	F	R	L	AC	C	P	N			
cc217		921493	64	F	C	R	AC	C	P	N			
cc219		921551	80	M	T	R	AC	B	P	N			
cc221	HIDE	924014	72	F	A	R	AC	C	N*	N		yes	yes
cc223	AGSC	924160	71	F	C	R	MUC	C	N	RER+	25	yes	yes
cc225		924223	80	M	C	R	AC	B	N	N			
cc227		924317	82	F	R	L	AC	A	P	N			
cc231	ANWO	924465	77	F	A	R	AC	B	P	N		yes	yes
cc235	ANKE	924634	83	F	A	R	AC	B	P	RER+	26	yes	yes
cc237	GEHA	924692	86	M	R	L	AC	B	N	N			yes
cc239		925332	70	M	R	L	AC	B	P	N			
cc243	CODR	St.Johns	41	M	R	L	AC	C	P	S1	27	yes	yes
cc245		925773	74	F	C	R	MUC	C	P	RER+	28		
cc247		926192	86	F	S	L	AC	B	P	N			
cc249		926223	79	F	C	R	AC	B	P	N			
	ANBA	94 16667	61	M	S	L	MUC	C	P	N		yes	yes
	ANSA	92 22249	73	F	LN	L	MUC	LN	P	N		yes	yes
	BEBR	9418950	92	M	S	L	MUC	B	P	N		yes	
	BUCH	94 18363	82	M	A	R	AC	C	P	N		yes	yes
	DUMA	91 01206	68	M	D	L	AC	B	P	N		yes	yes
	EDDO	91 17684	78	M	S	L	AC	B	P	N		yes	yes
	FRGA	9107239	73	M	C	R	MUC	B	N	RER+		yes	
	JAMU2	94 17252	85	M	A	R	AC	C	P	N		yes	yes
	JEDO	91 18129	68	F	S	L	AC	B	N	N		yes	
	LADI	9110174	39	M	S	L	AC	A	N	RER+		yes	
	MABO	9121073	42	F	R	L	AC	C	P	N		yes	
	MACA	94 18836	81	F	C	R	AC	B	P	RER+			yes
	SHCH	9425782	NA	F	NA	L	NA	NA	P	N		yes	
	WIMCF	9111273	28	M	R	L	AC	D	N	N		yes	
	AGMCK*	9116209	36	F	R	L	AC	B	P	S1		yes	
	THCU*	9216621	88	M	R	L	MUC	B	N	N		yes	
	VIWA*	9216621	46	F	R	L	AC	B	P	N		yes	

## Key to Appendix 5

### Column headings

Patient and tumour details are given in columns headed **age**, **sex**, tumour **site** and **side**, histological tumour **type** and **Dukes** stage. Patients and tumours referred to in chapters 3, 4 and 5 are indicated in columns headed **Chapter 3**, **Chapter 4** and **CGH**, respectively. **p53** refers mainly to IHC data unless otherwise indicated, and **shift** to RER status.

**Patient** and **Pathology reference no.** refer to departmental and hospital reference numbers, and are intended for future cross-reference; they are not directly relevant to this study.

### Site

C caecum  
A ascending colon  
T transverse colon  
D descending colon  
S sigmoid  
R rectum  
LN lymph node

### Side

L left  
R right

### Type

AC adenocarcinoma  
MUC mucinous carcinoma

### p53

N IHC negative  
P IHC positive  
N\* IHC negative but with evidence of p53 mutation

NA information not available

### Shift

N no shift (RER-)  
S1 shift at one locus only  
RER+ shift at more than one locus

## **PUBLICATIONS AND PRESENTATIONS ARISING FROM THIS THESIS**

Bubb, V.J., Curtis, L.J., Cunningham, C., Dunlop, M.G., Carothers, A.D., Morris, R.M., White, S., Bird, C.C. and Wyllie, A.H. Microsatellite instability and the role of hMSH2 in sporadic colorectal cancer. *Oncogene* 12: 2641-2649 (1996).

Curtis, L.J., Bubb, V.J., Cunningham, C., Dunlop, M.G., Carothers, A.D., Morris, R.M., White, S., Bird, C.C. and Wyllie, A.H. Microsatellite instability and the role of hMSH2 in sporadic colorectal cancer. Poster presentation, American Association for Cancer Research Special Conference, 'Cancer Susceptibility Genes and Molecular Carcinogenesis', Keystone, Colorado, February 19-25, 1996.

Curtis, L.J., Scheler, J., Doig, J., White, S. and Wyllie, A.H. Detection of chromosome abnormalities in sporadic colorectal cancer by comparative genomic hybridisation. Oral presentation at Pathological Society meeting, London, January 1997.



# Microsatellite instability and the role of hMSH2 in sporadic colorectal cancer

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Microsatellite instability (MSI) occurs in most tumours of patients with hereditary non-polyposis colorectal cancer (HNPCC) and in around 17% of sporadic colorectal cancers. Germline defects in mismatch repair (MMR) genes are responsible for the majority of large HNPCC families, with hMSH2 accounting for at least 30%. MMR gene defects also occur in a small proportion of sporadic colorectal tumours with MSI. We report a systematic analysis of mismatch repair deficiency in 215 Scottish patients with sporadic colorectal tumours. We found that 16.4% of tumours showed MSI; survival analysis by Cox proportional hazards method showed a substantial survival advantage for patients with tumours showing MSI, independent of other prognostic factors. Tumours with MSI were found for hMSH2 mutations and although 61% were found to have alterations, of these only 1/24 was exonic. The majority of these changes were reductions in length of mononucleotide tracts and we postulate that these alterations are the result of a genetic defect in hMSH2, although they may compromise hMSH2 function as a second step in tumorigenesis. Our findings suggest that instability confers an improved prognosis in colorectal cancer and, despite the fact that these two types of tumours share similar biological characteristics, the genetic basis of HNPCC and sporadic colorectal cancer with MSI is different.

**Keywords:** replication errors; sporadic colorectal cancer; hMSH2; prognosis

## Introduction

Tumours from hereditary non-polyposis colorectal cancer (HNPCC) patients are characterised by the presence of multiple replication errors (Ionov *et al.*, 1993; Aaltonen *et al.*, 1993). As a consequence of a defect in the mismatch repair pathway in such patients, mutations are unrepaired. Within microsatellite sequences such replication slippage is manifest as variation in length, termed microsatellite instability (MSI). MSI has also been demonstrated in sporadic colorectal cancers (Thibodeau *et al.*, 1993) and frequently in many other cancers, including pancreatic (Han *et al.*, 1993), gastric (Han *et al.*, 1993; Nakai *et al.*, 1993; Mironov *et al.*, 1994; Rhyu *et*

*al.*, 1994; Chong *et al.*, 1994), prostatic (Gao *et al.*, 1994), endometrial (Risinger *et al.*, 1993; Peltomäki *et al.*, 1993), breast (Patel *et al.*, 1994), non-small cell lung cancers (Shridhar *et al.*, 1994; Fong *et al.*, 1995) and small cell lung cancers (Merlo *et al.*, 1994) and in Muir-Torres syndrome (Honchel *et al.*, 1994).

Up to 86% of tumours from HNPCC patients exhibit instability at multiple microsatellite sites (Aaltonen *et al.*, 1993, 1994; Wu *et al.*, 1994). A significant, although much lower, proportion (in most studies around 17%) of sporadic colorectal carcinomas show shifts in allele size in at least one microsatellite locus relative to their corresponding normal tissue (Aaltonen *et al.*, 1993, 1994; Ionov *et al.*, 1993; Lothe *et al.*, 1993; Young *et al.*, 1993; Kim *et al.*, 1994), although others have found the frequency to be as high as 28% in the population studied (Thibodeau *et al.*, 1993). The prevalence of tumour MSI is greater when cases with some family history of cancer are included (Lothe *et al.*, 1993). Sporadic tumours with microsatellite instability have characteristics in common with HNPCC tumours. They are usually located in the proximal colon (Ionov *et al.*, 1993; Thibodeau *et al.*, 1993; Lothe *et al.*, 1993; Aaltonen *et al.*, 1993; Kim *et al.*, 1994), associated with extracellular mucin production (Kim *et al.*, 1994), poor differentiation (Ionov *et al.*, 1993; Lothe *et al.*, 1993; Kim *et al.*, 1994) and diploidy (Aaltonen *et al.*, 1993; Lothe *et al.*, 1993). They also show a negative correlation with mutation (Ionov *et al.*, 1993) or immunocytochemical stabilisation of p53 (Kim *et al.*, 1994) and Ki-ras mutation (Ionov *et al.*, 1993). In addition, loss of heterozygosity at known tumour suppressor gene loci on chromosomes 5q, 17p and 18q is relatively less frequent in tumours with MSI compared to those without (Thibodeau *et al.*, 1993). Finally, these tumours show a tendency towards increased patient survival (Thibodeau *et al.*, 1993; Lothe *et al.*, 1993).

Recently, a number of genes that participate in human mismatch repair have been identified, namely hMSH2 (Fishel *et al.*, 1993; Leach *et al.*, 1993), hMLH1 (Bronner *et al.*, 1994; Papadopoulos *et al.*, 1994), hPMS1 and hPMS2 (Nicolaidis *et al.*, 1994) and p160/GTBP (Palombo *et al.*, 1995; Drummond *et al.*, 1995; Papadopoulos *et al.*, 1995). The roles of these genes were inferred from their homology with bacterial and yeast mismatch repair genes (Prolla *et al.*, 1994), and germ-line mutations of all except p160/GTBP have been found in HNPCC families. Defects in the mismatch repair pathway account for the vast majority of HNPCC families; mutations in hMSH2 are thought to account for at least 50% of kindreds

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(Liu *et al.*, 1994) and mutations in hMLH1 for up to 33% (Han *et al.*, 1995; Liu *et al.*, 1996). Mutations in these genes may also play a role in the development of sporadic colorectal cancer. However, mutations in mismatch repair genes have so far been demonstrated in only a small percentage of sporadic cancers or cell lines derived from colorectal carcinomas (Liu *et al.*, 1995; Borresen *et al.*, 1995).

We sought to clarify further the prevalence of mismatch repair deficiency in a large unselected series of 215 Scottish sporadic colorectal cancer cases. We confirm the previously reported characteristics of such tumours: their predilection for origin in the proximal colon, tendency for mucinous histology, negative correlation with abnormalities in p53 expression and substantially better prognosis than tumours in patients without MSI. To delineate the role of a specific mismatch repair gene known to be important in hereditary cancers sharing these characteristics, we carried out an extensive mutation analysis of hMSH2 on selected cases. Our results revealed a substantial difference in the nature and incidence of mutations in this gene between sporadic and hereditary (HNPCC) colorectal cancers, emphasising that the mechanism underlying tumourigenesis is different in these two groups.

## Results

### Microsatellite instability analysis

We analysed 215 patients diagnosed as having primary sporadic colorectal cancer. This group included patients with more than one tumour and those with cancer at other sites (either synchronous or asynchronous) who did not fit the criteria for HNPCC (Percepe *et al.*, 1994). When patients had more than one carcinoma, each lesion was analysed separately if DNA was available. Thus, a total of 219 carcinomas were analysed, four patients each having two synchronous cancers.

These 219 cancers were staged as follows: 25 Duke's Stage A, 100 Duke's Stage B and 94 Duke's Stage CD (for these purposes stages C and D were amalgamated as information regarding distant metastasis was not always available). The average age was 70 years, range 28–95 years, with one patient of unknown age. The group comprised 46.5% (100/215) males and 53.5% (115/215) females.

All samples were analysed for genetic instability at a minimum of four microsatellite loci. 16.4% (36/219) exhibited instability at one or more loci. The average age of this group was 68.75 years, range 37–93 years. This is not significantly different from the age of the group without evidence of MSI (70.02 years, range 28–95). During the course of this study we identified a polyadenine tract located in the 5' region of hMSH2 intron 5 which exhibited replication errors in 58% (19/33) of samples showing microsatellite instability at other loci. When instability at this locus was included, we identified three additional patients with evidence of MSI, one of which appeared to show instability at another locus but despite repetition had not given an unequivocal result. Thus, in total, 17.8% (39/219) of sporadic colorectal cancers in this series exhibited MSI.

When we applied the more stringent requirement of instability at two or more loci (including intron 5 of hMSH2), a criterion often used to define a replication error positive (RER+) phenotype (Aaltonen *et al.*, 1993; Parsons *et al.*, 1993), the incidence of MSI was 10.5% (23/219).

The relationship between microsatellite instability and site of tumour, p53 stabilisation and Duke's stage was examined (Table 3). We found 59% (23/39) of lesions with instability at one or more loci were in the proximal or right side of the bowel (ie proximal to and including the splenic flexure). This was significantly different ( $\chi^2=7.73$ ,  $P<0.01$ ) from the distribution of those without MSI (non-shifters), where 35% (63/180) were right-sided, similar to the general distribution of all colorectal cancers in the UK of which 28% are right-sided (Cancer Research Campaign, 1993). Of lesions with at least two unstable sites, 74% were proximal ( $\chi^2=12.93$ ,  $P<0.001$ ). When proximal tumours alone were considered, 27% (23/86) demonstrated microsatellite instability at one or more loci, as compared with only 12% (16/133) of distal cancers.

When we looked for evidence of p53 stabilisation in our series [scored positive if any tumour nucleus in the section stained (Purdie *et al.*, 1991)] 56.4% (22/39) of tumours with MSI at one or more loci stained positively for p53 protein, which was not significantly different from those without MSI, in which 60% (108/180) had evidence of stabilised p53 ( $\chi^2=0.43$ ,  $P>0.5$ ). However, a significant inverse relationship was seen between MSI status and p53 staining in tumours with instability at more than one loci ( $\chi^2=4.43$ ,  $P<0.05$ ). Fifty-nine percent of our total population had positively stained nuclei. When the site of lesion was considered 54% of proximal tumours and 64.5% of distal tumours had evidence of stabilised p53. We compared these results to data generated from the analysis of a subset of these cases for loss of heterozygosity (LOH) at 17p using three restriction fragment length polymorphic (RFLP) markers intragenic to p53 (Purdie *et al.*, in preparation). 125 lesions were analysed for intragenic loss of p53, of which 49 were informative at one or more loci. Of these informative cases, 19 had allele loss involving p53 and in three (16%) of these we demonstrated MSI, all located on the left side of the bowel. Of the 30 lesions where heterozygosity in p53 appeared to be retained, nine (30%) exhibited MSI; of these, six were located on the right side of the colon. Thus there is an apparent trend for tumours that exhibit MSI to retain heterozygosity in p53, but this difference was not statistically significant. There was no significant difference between Duke's Stage and MSI status in tumours with MSI at one or more loci ( $\chi^2=2.37$ ,  $P>0.3$ ) or at more than one loci ( $\chi^2=2.028$ ,  $P>0.3$ ) (Table 3), although lesions with MSI appeared to be less common within the Duke's A group.

Histological examination of the 39 tumours with MSI revealed that 41% (16/39) of these tumours were mucin-secreting. This is a much higher proportion than in the unselected Scottish population of sporadic colorectal cancers; in such a series overlapping with the present study we observed only 17% (59/346) mucinous carcinomas.

# al analysis

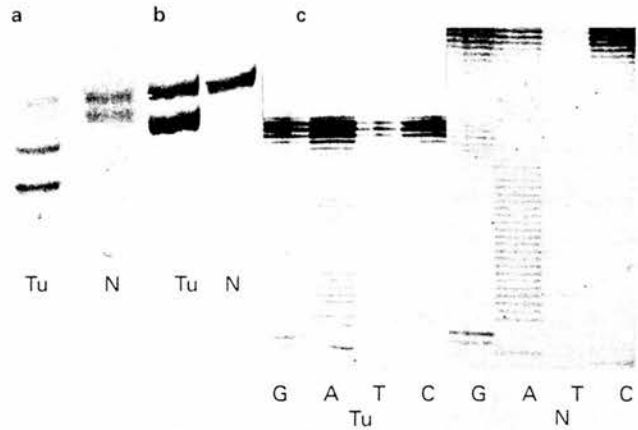
al analysis was carried out on 169 patients for information was available on all factors (46 ed) using the Cox proportional hazards method. Most significant predictor of survival was Duke's ( $\chi^2=38.4$ ,  $P<0.0001$ ) followed by age ( $\chi^2=9.11$ ,  $P=0.025$ ), MSI ( $\chi^2=7.83$ ,  $P=0.0051$ ) and side ( $\chi^2=3.35$ ,  $P=0.012$ ). The hazard ratio of patients tumours showing MSI to those without was estimated to be 0.39 (95% C.I. 0.19–0.82). Thus, at point in time after diagnosis, a patient whose tumour showed MSI had a risk of dying which was estimated to be about 39% of that of a patient with a tumour without MSI, even after allowing for the influence of other predictive factors. The results are presented as an estimated survival curve (Figure 3). Effects of p53 immunohistochemical status and sex were unrelated to prognosis.

In order to eliminate any possibility that tumour side was a consequence of MSI status the test was repeated using 'side'. The result showed negligible influence on predicted survival of patients whose tumours had MSI compared to those without [hazard ratio of 0.37 (95% C.I. 0.17–0.77)]. No evidence was found of interactions between Duke's stage, age, MSI status, side and sex.

## ion analysis of hMSH2

We analysed 36 tumours with microsatellite instability at one or more loci (including hMSH2 intron 5) for mutations in the hMSH2 gene (Table 4). In addition, 36 tumours without MSI were analysed as controls. We found two tumours which each had a single exonic mutation. Both of these were mis-sense mutations and both occurred in the control group. One tumour with MSI was found to carry any mutation, a frameshift creating a new stop codon. This tumour also showed a reduction in length of polyadenine tract located three nucleotides upstream of the 3' end of exon 5, a change which was found in an additional 21 tumours. Of these 21 tumours the length change was somatic in 20 cases. The alterations were initially detected by abnormal banding on SSCP gels, but were later found to be detectable on denaturing polyacrylamide gels. Normal allele lengths ranged from 10 to 23 bases compared with matching normal tissue which had 25 nucleotides in this tract, which differs from the previously published sequence of 26 residues (Liu *et al.*,

1994). In a further tumour with MSI had an intronic mutation, an alteration of unknown function, as well as a length reduction in intron 5 and a second mutation in the length of intron 1. Seven other tumours had this somatic reduction in the length of polythymine tract located 3' in intron 1, predicted to be part of the splice site. Whereas in normal tissue the tract extends for 12 or 13 nucleotides, this was reduced to as few as nine base pairs in some tumours. Analysis of cancer-free control samples demonstrated that 12 or 13 bases was a normal polymorphic variation. Removal of this polynucleotide tract by digestion and repetition of SSCP on the residual polynucleotide fragment (containing 90% of the exonic sequence) revealed no further mutations.



**Figure 1** Intron 5 polyadenine tract length change in one patient (patient 19 in Table 4) shown by (a) SSCP, (b) heteroduplex and (c) sequencing analysis (nucleotide lanes labelled). In each analysis, tumour DNA (Tu) and normal DNA (N) are shown for comparison

When these results are combined, 15 tumours had a single change in the hMSH2 gene, eight tumours had two alterations and one had three. Thus, a total of 61% (22/36) of tumours with MSI had an alteration in hMSH2 compared to 6% (2/32) of non-shifters analysed. Forty-six percent (11/24) of tumours with hMSH2 mutations showed mucinous histology.

## Polymorphisms within the hMSH2 gene

Two alleles were detected by heteroduplex analysis in intron 1, which sequencing revealed to be a C→G substitution at the +9 position. This destroyed a *BcnI* cleavage site, permitting use of a PCR–RFLP assay to calculate allele frequency in 106 unselected healthy blood donors. The allele frequencies were A1=0.38 and A2=0.62 in this local population. The same frequency for each allele was found for the cases analysed for hMSH2 mutation in our study population. Thus this polymorphism does not appear to influence susceptibility to sporadic colorectal cancer.

Twenty-seven percent of patients with MSI were heterozygous for a previously described polymorphism in intron 12 (Fishel *et al.*, 1993; Hall *et al.*, 1994). Two patients carried both the polymorphism described at +12 position of intron 10 (Wijnen *et al.*, 1994) and the polymorphism described at –9 position of intron 9, GTCGTT-GTCATT (Børresen *et al.*, 1995).

## Discussion

This study has identified a group comprising a sixth of a population of Scottish sporadic colorectal cancer patients that exhibit microsatellite instability. This proportion is in agreement with the incidence of MSI identified by others in sporadic CRC from other geographic locations, including Lothe *et al.* (1993) (16.5%); Aaltonen *et al.* (1993) (13%) and Kim *et al.* (1994) (13%). Our data include a number of cases where only one locus is altered, a phenomenon which has been previously noted in colorectal cancer (Peltomäki *et al.*, 1993; Lothe *et al.*, 1993).



We examined other clinico-pathological factors that may distinguish this group from the general population of colorectal cancer patients. Although we found no significant difference between microsatellite unstable and stable tumours for age of onset, Duke's Stage, or gender distribution, the data did confirm the proximal location of colonic lesions with MSI which has been noted by others (Lothe *et al.*, 1993; Kim *et al.*, 1994; Thibodeau *et al.*, 1993). Additionally, we found that there was a significant inverse relationship between p53 stabilisation and MSI status in tumours with instability at more than one loci. This relationship has also been reported by others (Ionov *et al.*, 1993), although without indication whether the tumours analysed were sporadic or hereditary in nature. Moreover, we find that MSI tumours are about twice as common within the group of tumours which have retained both alleles of p53.

The results of the Cox analysis indicate a highly significant favourable effect of MSI status on post-operative survival, even after allowing for the effects of age, Duke's stage, sex and tumour side. Paradoxically, histological features generally associated with aggressive tumour behaviour are common in MSI tumours. In our series 41% are mucin-secreting. The frequency is 46% in tumours with a hMSH2 mutation. In addition, a higher proportion of poorly differentiated tumours associated with MSI has been reported by others (Ionov *et al.*, 1993; Lothe *et al.*, 1993; Kim *et al.*, 1994). It has been suggested that this survival advantage stems from an enhanced host immune response to cancer cells arising as a result of a high mutation rate of tumour-associated antigens (Kim *et al.*, 1994). Alternatively perhaps the situation is reached where the tumour can no longer survive with such a mutation burden if, for example, it compromises the function of essential housekeeping genes. Recent insights into the mechanism of development of tumours with MSI suggest that many of these carcinomas have inactivation of the TGF $\beta$  receptor, rendering cells insensitive to TGF $\beta$ -mediated growth inhibition (Markowitz *et al.*, 1995). Thus, determination of the MSI status of a colorectal lesion could be important not only as a predictor of prognosis but also in terms of potential cancer therapy.

Although sporadic colorectal cancers with MSI demonstrate replication slippage in a similar manner to HNPCC lesions and share similar biological characteristics, there are clear differences. Unlike the situation seen in sporadic colorectal cancer, microsatellite instability occurs as an early event in HNPCC adenomas (Young *et al.*, 1993; Aaltonen *et al.*, 1993), as it does in Barrett's oesophagus, oesophageal adenocarcinoma (Meltzer *et al.*, 1994) and ulcerative colitis (Suzuki *et al.*, 1994). The role of hMSH2 is clearly different in HNPCC and sporadic colorectal cancer. Only 3% (1/36) of unstable tumours and 6% (2/32) of stable tumours had one or more mutation in the exonic portions, splice acceptor or donor regions of hMSH2. This concurs with the results of the analysis of a similar series of sporadic tumours (Børresen *et al.*, 1995) and is akin to the incidence of hMSH2 mutation in 20 cell lines derived from sporadic colorectal cancers with MSI (Liu *et al.*, 1995). However, it is in direct contrast to the situation found in HNPCC where analysis of these regions identifies a mutation incidence of up to 40% (Liu *et al.*, 1994; Wijnen *et al.*, 1995).

We have, however, shown many intronic defects within the hMSH2 gene, regions which to our knowledge have not been examined extensively for mutations in HNPCC tumours. Introns are likely potential targets for mutation because they contain repetitive sequences and the observed alterations in length of polynucleotide tracts would be consistent with a primary defect in another mismatch repair gene. Instability of polyadenine tracts in colorectal cancer, this time in random stretches of DNA, has been demonstrated by others (Chen *et al.*, 1995) and it is interesting to note that the mutation spectrum associated with a recently discovered hMSH2 binding protein, GTBP or p160, tends towards reductions in length of mononucleotide repeated units such as this (Papadopoulos *et al.*, 1995). The effect on gene function of these intronic alterations within the hMSH2 gene is as yet unknown. We postulate that they may compromise transcriptional efficiency of hMSH2, but at this time we cannot predict whether this would act globally or have a local effect by a mechanism such as exon skipping. Unfortunately RNA is not available from these lesions to fully address this point. There is evidence that mutations of this type within or outwith splice sites can cause inefficient or aberrant splicing (Wieringa *et al.*, 1984; Chu *et al.*, 1993). Thus, these hMSH2 defects might contribute to the development of a proportion of sporadic colorectal cancers through further crippling mismatch repair.

The possible impact of double and triple mutations in hMSH2 is unclear. It is thought that both alleles of hMSH2 are inactivated, like a classical tumour suppressor gene, during the development of colorectal cancer. However others have shown a dose-response effect of this gene during *in vitro* studies (Parsons *et al.*, 1993). It is also not yet known if all missense mutations described inactivate the gene to the same degree, which could account for phenotypic variation. Unlike the situation seen with other tumour suppressor genes, loss of heterozygosity is not commonly observed at the hMSH2 locus (Aaltonen *et al.*, 1993).

The overall incidence of hMSH2 mutation in our population of sporadic colorectal cancers is at least 11%, with only 1% having exonic mutations. Amongst our population of patients with apparently sporadic colorectal cancer we found three who carried germ-line mutations in hMSH2. This small subset of patients may be thought to represent hereditary, rather than sporadic, colorectal cancers which do not fit the criteria for HNPCC. Only one of these three patients had any family history of cancer. Interestingly, this patient was identified from the group of cancers without MSI. He was younger (49 years) than our average age-group, had a Duke's C carcinoma in the ascending colon, and had a family history of pancreatic cancer. This individual may belong to the class of familial RER<sup>+</sup> tumours including lung, breast and pancreas, with less prevalent microsatellite alterations (Liu *et al.*, 1995) although we are unaware that this has previously been associated with hMSH2 mutation. A second patient with no family history or evidence of microsatellite instability, aged 74 and with a cancer of the caecum, was also found to have a germ-line hMSH2 mutation. Both of these mutations were missense and therefore predicted to compromise protein function. The third germline mutation was the shortened polyadenine tract

on 5 of unknown function: this was found in a  
ir demonstrating MSI. These observations sup-  
he view that a wide range of phenotypes are  
ated with germ-line hMSH2 mutation (Kolodner  
1994; Mary *et al.*, 1994).

o major conclusions can be drawn from this  
Firstly, although MSI is clearly a significant  
in the clonal expansion of around one in six of  
sporadic colorectal cancers, exonic hMSH2 defects  
associated with this instability in a considerably  
proportion of tumours than in HNPCC. Indeed,  
mutations in the intronic sequences of hMSH2  
cluded from consideration, hMSH2 mutation in  
dic tumours with MSI is no more frequent than  
er tumours. Our study therefore suggests that the  
c basis of microsatellite instability in sporadic  
nherited colorectal cancer is different. This is  
rted by evidence of a dramatic difference between  
cidence of MSI in adenomas from HNPCC and  
dic colorectal cancer patients (Young *et al.*, 1993;  
nen *et al.*, 1994). Secondly, our data (from a large  
of patients with extensive follow-up) clearly  
strate a survival advantage for patients who  
a tumour exhibiting MSI. It will be of great  
st to determine the mechanism by which such  
c instability imparts improved prognosis since  
may have substantial implications for novel  
euthetic approaches.

## als and methods

### samples

s were harvested fresh from consecutive colorectal  
omas removed at operation between 1988–1993, and  
at –70°C. DNA was extracted from paired normal  
olved mucosa or blood) and tumour tissue by the  
d of Goelz *et al.* (1985). Representative portions of  
r were paraffin-processed following standard metho-  
y.

### erage chain reaction (PCR) of microsatellite sequences

umours were analysed for evidence of genetic  
ility at four microsatellite loci, D2S119, D3S1293,  
2 and D13S160 (Gyapay *et al.*, 1994). In addition,  
were examined at loci D15S132 and D17S849 by  
ated Laser Fluorography (ALF, Pharmacia Biosys-  
td.). Amplification conditions were 20 mM Tris-HCl  
4), 50 mM KCl, 200 µM each dNTP, 0.5 µM each

primer (Table 1), 2.5 units *Taq* DNA polymerase (Life  
Technologies) in 100 µl reaction and 100 ng genomic DNA  
template. Magnesium ion concentrations for each primer  
set are indicated in Table 1. The upstream primer of each  
primer pair was synthesised with a fluorescein molecule at  
the 5' position to allow analysis by ALF.

### Analysis of microsatellite marker sequences

For silver staining, products were heat denatured in 0.5  
volume STR loading buffer (10 mM NaOH, 95% forma-  
mide, 0.05% bromophenol blue, 0.05% xylene cyanol FF)  
and electrophoresed on 6% 19:1 acrylamide:bis acryla-  
mide denaturing gels containing 7 M urea. Electrophoresis  
buffer was 44.5 mM Tris, 44.5 mM Boric acid, 1 mM  
EDTA, pH 8.3 and gels were electrophoresed at constant  
power (70 W). To facilitate silver staining the gel was  
bound to the glass plate with γ-methacryloxypropyl-  
trimethoxysilane (Kobayashi, 1988; Storts *et al.*, 1993)  
and stained *in situ* according to the method of Bassam *et al.* (1991).

PCR samples analysed by ALF were prepared by adding  
an equal volume of denaturing solution [95% formamide,  
0.8% dextran blue and 25 mM EDTA (pH 9)] and heat  
denatured at 95°C for 5 min, then rapidly cooled on ice.  
Internal standards between 65 and 313 nucleotides long, were  
prepared by amplification from single stranded M13mp18  
vector using a variety of 20–24mer primers. Five microlitres  
of PCR product in denaturing solution were loaded onto a  
6% MDE gel (AT Biochem, PA, USA) containing 7 M urea  
and electrophoresed at constant power (55 W) and tempera-  
ture (50°C). Evidence of microsatellite instability was  
determined by a shift of allele peak to a new position.

### Polymerase chain reaction of exons from hMSH2

Each of the 16 exons of hMSH2 was amplified using the  
primers described in Table 2. PCR was carried out for 30  
cycles at annealing temperatures calculated according to  
primer composition. Reactions consisted of 50 µl volume  
containing 100 ng genomic DNA, 12.5–25 pmoles of each  
primer, 200 µM of each dNTP (Pharmacia Biosystems Ltd),  
2.5 mM MgCl<sub>2</sub> (except exons 15 and 1, which contained  
1.5 mM), 1.25 U of thermostable DNA polymerase and  
buffer consisting of 10 mM Tris-HCl (pH 8.8 at 25°C),  
50 mM KCl and 0.1% non-ionic detergent.

### Mutation analysis of hMSH2 gene

Single stranded conformational polymorphism (SSCP)  
analysis was carried out as previously described (Curtis *et al.*,  
1994). Heteroduplex analysis was also performed on  
those samples in which the banding patterns proved  
difficult to resolve by SSCP. PCR products for hetero-

Table 1 Microsatellite loci primers

Marker name	Locus	[Mg <sup>2+</sup> ]	Primer
AFM077yb7	D2S119	2.0 mM	CTTGGGGAACAGAGGTCATT GAGAATCCCTCAATTTCTTTGGA
AFM200zal	D3S1293	2.0 mM	ACTCACAGAGCCTTCACA CATGGAAATAGAACAGGGT
AFM234vf4	D8S282	1.5 mM	GGGCACAGGCATGTGT GGCTGCATTCTGAAAGGTTA
AFM157xa11	D13S160	1.5 mM	CGGGTGATCTAAGGCTTCTA GGCAGAGATATGAGGCAAAA
AFM265xf9	D15S132	1 mM	CTGATAATAAAACCAGGAAGACAC TATTGGCCTGAAGTGCTG
AFM234wg3	D17S849	1 mM	CAATTCTGTTCTAAGATTATTTTGG CTCTGGCTGAGGAGGC

Table 2 hMSH2 primers

Exon	Primers (5' to 3')
1	GCGCATTTCCTTCAACCAGG AAAGGAGCCGCGCCACAA
2	ACATGTAATATCTCAAATCTG AAATAAGTAAATTAAAAAGGAAG
3	AAAGTATGTTCAAGAGTTTGT ATCTCCTCTATCACTAGACT
4	TTATTCCTTTTCTCATAGTAG TTGTAATTCACATTTATAATCC
5	TGGCTATAGGAAATCTTCG AACCATTCAACATTTTTAACC
6	ACTAATGAGCTTGCCATTC AACTGCAGGTTACATAAAAC
7	AAATATTTTACATTAATTCAAG GTATGIGTTGAAGGAAAAC
8	GATCTTTTATTTGTTTG AAAAATAACTACTGCTTA
9	GGATTTTGTCACTTTGTTC TAGGACAAAAGAATTATTCC
10	TGGTAGTAGGTATTTATGG CATTTAGGGAATTAATAAAG
11	ACTGTTATTTTCGATTTGCAG CCAGGTGACATTCAGAAC
12	TTTATTATTCAGTATTCCTGTG CCACAAAGCCCAAAAACCA
13	AGAAAGAAGTTTAAACTTGC CTATCTTCAAGGGACTAGG
14	TACCACATTTTATGTGATG TACTGAATTTAGAGTACTC
15	GCTGTCTCTTCTCATG TAATAATAGAGAAGCTAAG
16	AGAIATTTTAAATTACTAATGGG AATATCTTATTAAGTTGATAGC

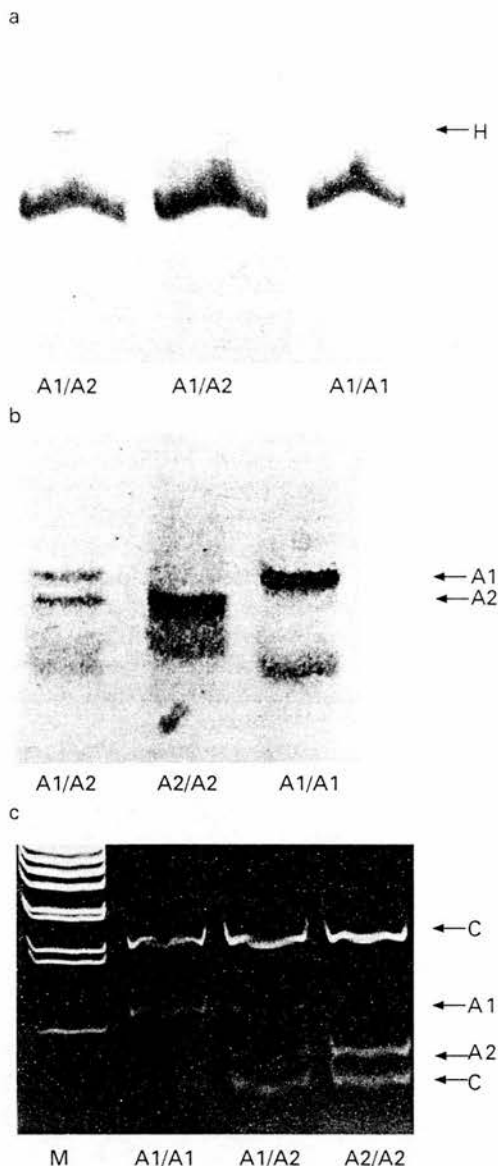


Figure 2 Exon 1 polymorphism, C to G change at the +9 position of intron 1, in normal DNA from three patients of different genotype by (a) heteroduplex, where H indicates heteroduplex formation (b) SSCP and (c) *BclI* restriction digest analysis. Allele sizes for *BclI* digestion products are 81 bp (A1) and 57 bp (A2). C indicates the constant bands of 152 bp and 48 bp (21 bp band not shown); M, DNA molecular weight marker 1 kb ladder (Life Technologies, UK)

Table 3 Characteristics of tumours with MSI

	Unstable $\geq$ 1 locus	MSI status Unstable > 1 locus	Stable <sup>a</sup>
Side			
Left	16 (41%)	6 (26%)	117 (65%)
Right	23 (59%)	17 (74%)	63 (35%)
Dukes stage			
A	2 (5%)	1 (4.5%)	23 (13%)
B	21 (54%)	13 (56.5%)	79 (44%)
CD	16 (41%)	9 (39%)	78 (43%)
p53 Status			
ICC Positive <sup>b</sup>	22 (56%)	9 (39%)	108 (60%) <sup>d</sup>
ICC Negative	17 (44%)	14 (61%)	66 (37%)
Unknown <sup>c</sup>			6
Total	39	23	180

<sup>a</sup>no alteration in length seen at loci tested. <sup>b</sup>immunocytochemistry. <sup>c</sup>PLPD fixed tissue unavailable. <sup>d</sup>Three samples included with strong cytoplasmic staining, nuclei were not stained

duplex analysis were heated to 95°C and cooled slowly to 37°C over 30 min. 5 µl of sample in 1 µl loading buffer (30% glycerol, 0.25% bromophenol blue, 0.25% xylene cyanol FF) was electrophoresed on a MDE gel containing 3.12 M urea according to the manufacturer's protocols (AT Biochem, PA, USA) and run at 0.71–0.8 kV for 16 h. Both glass plates were pre-treated, one with Gel Slick (AT Biochem, PA, USA) and the other with 20 µl γ-methacryloxypropyltrimethoxysilane in 5 ml distilled water (pH 3.5 with acetic acid). Silver staining was performed according to the following protocol: The gel was soaked for 10 min in 10% ethanol then 10 min in 1% nitric acid, rinsed quickly with water and washed for 5 min in water. It was then soaked for 20 min in 12 mM silver nitrate, rinsed twice for 20 s, first with water then with cold developer (0.28 M sodium carbonate, 0.0185% formaldehyde) until a precipitate formed. The developer was replaced and the gel soaked until an image appeared. The reaction was stopped with 0.1 M citric acid and the gel rinsed in water. The whole procedure was carried out on a rocking platform.

Twenty-one products generated from exon 5 were abnormal by SSCP and sequencing revealed changes in the length of a polyadenine tract within the intron. In order to eliminate the possibility of other mutations in this exon, this portion was cleaved away with *TruI* (MBI Fermentas, Lithuania) and the remaining exonic fragment reanalysed by SSCP. Similarly, eight products generated from exon 2 which were abnormal by SSCP but revealed only a length change in an intronic polynucleotide tract by sequencing, were cleaved with *PstI* to allow reanalysis of the exonic portion by SSCP.

Samples which demonstrated an abnormal banding pattern by heteroduplex or SSCP analysis were reamplified with *Taq* polymerase and directly sequenced (Sequenase PCR Products Sequencing Kit, United States Biochemicals) or cloned into pGEM7ZF+ (Promega Ltd., UK) and sequenced as pooled clones (20–100 colonies) using a modified dideoxy



Table 4 hMSH2 mutations

no	Exon-intron	Codon	Mutation type	Mutation genotype	MSI status
	1	codon 21 CAC→CAG His-Gln	mis-sense	germ-line	stable
	2	T→C, -19 position of intron 1 3' end	intronic	somatic	shift
	3	codon 131 AAT→AGT Asn-Ser	mis-sense	germ-line	stable
	15	codon 847 deleted T, creates stop codon downstream	nonsense	somatic	shift
28,53, .89	2	reductions in polythymine tract length, 3' end intron 1	intronic	somatic	shift
9,26,28,34, .51,53,61,62, .82,84,85, .89,	5	reductions in polyadenine tract length, 5' end intron 5	intronic	somatic	shift
				germ-line	shift

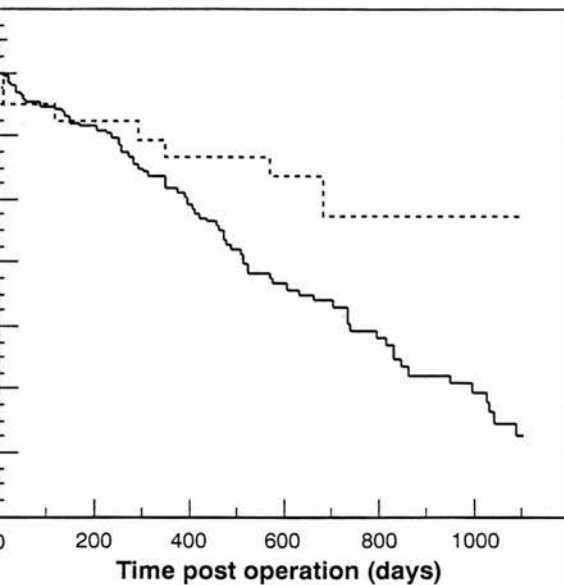


Fig. 3 Estimated survival probabilities for patients with MSI and those without (—) by days post-operation using the proportional hazards method. Probabilities are plotted for a hypothetical set of patients with the same mean effects of Duke's tumour side and age as observed in our sample. The number of patients with and without MSI surviving at 1100 days was six and 29 respectively

termination method (Sequenase version 2.0, United Biochemicals) with the appropriate PCR primer. PCR fragments did not produce high quality sequence by this method, single colonies were sequenced with the T7 promoter primer (Promega Ltd., UK). Due to the low fidelity of *Taq* enzyme, sequence data from single colonies generated by *Taq* polymerase were confirmed using alternative polymerase, *rTth* DNA polymerase (Perkin Elmer, UK), which has proofreading activity and a significantly lower error rate. These samples were cloned into pCR11 using the TA cloning kit (Invitrogen BV, Leek, Holland) and single colonies sequenced. A frequent abnormality in the exon 1 PCR fragment was identified by the presence of an extra band by heteroduplex analysis and a consistent SSCP banding pattern (Figure 2). This additional allele was identified by sequence analysis and its status confirmed by restriction digestion (*BclI*) followed by electrophoresis on a 20% polyacrylamide gel.

In addition, the incidence of a previously reported polymorphism in intron 12 (Fishe *et al.*, 1993; Hall *et al.*, 1994) was investigated. A restriction digestion assay was developed which included digestion by *SfcI* followed by electrophoresis on a 3% agarose gel.

#### Immunocytochemical staining of p53 protein

Representative portions of tumour were fixed in periodate-lysine-paraformaldehyde-dichromate (PLPD) and embedded in paraffin wax. Staining was performed with the monoclonal anti-p53 antibody, Pab1801 (Oncogene Science, UK) as previously described (Purdie *et al.*, 1991). Samples were scored as positive if any positive nuclei were found in the section and were analysed by at least two observers.

#### Statistical analysis

The effect on post-operative survival of all relevant factors was investigated by analysis with the Cox proportional hazards method, using the Cox Regression routines of SPSS for Windows 6.0. Post-operative survival was established by perusal of death certificates held by the Registrar General for Scotland. Those patients who did not appear in the registry on the census date were traced through hospital records to confirm survival by continuing out-patient attendance or by letter to their family practitioner. The censoring date for survival was taken as 31.12.93; thus, all who survived later than this date were treated as 'last known alive' at this time. Factors investigated as possible predictors of survival were age, tumour side, ploidy, p53 status, gender, shift status and Duke's stage. Deaths from all causes were considered.

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